A Dissertation for the Degree of Doctor of Philosophy

Pandemic influenza virus, H1N1, induces asthmatic symptoms and the development of indirect ELISA for canine influenza virus, H3N2

인플루엔자(H1N1) 감염에 의해 유발되는 천식증상과 개 인플루엔자, cH3N2, 특이항체 진단을 위한 간접효소면역측정법에 대한 연구

2015년 2월

서울대학교 대학원

농생명공학부

심 두 희

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February 2015

Department of Agricultural Biotechnology
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지도교수 유상렬

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Doo Hee Shim
(Supervised by Prof. Sangryeol Ryu)

Abstract

Influenza infection causes acute respiratory inflammation in human beings. Newly emerged strains of influenza A/California/04/2009 (H1N1) is the first and highly pathogenic flu pandemic of the 21st century. The pandemic strain of the influenza A virus (pH1N1) in 2009 caused many complications, including the induction of asthmatic symptoms in many patients. The mechanisms underlying asthmatic symptoms in pH1N1-infected patients remain to be elucidated. Several reports show that influenza virus infection causes asthmatic symptoms in mice. However, there is no report about airway hyperresponsiveness (AHR) in patients who developed H1N1 induced acute asthmatic symptoms without a previous history of asthma.

First part of this study performed to quantify asthmatic symptoms in pH1N1-infected children and elucidate the underlying mechanisms of airway hyperresponsiveness (AHR) induced in a murine model of pH1N1 infection. There is no report about the relationships between asthmatic symptoms and pH1N1 infection both in patients without previous history of asthma and in mice. To quantify asthmatic symptoms in pH1N1-infected children, we administered a methacholine challenge test (MCT) in confirmed pH1N1-infected children who were hospitalized between October 2009 and February 2010 with moderate to severe acute asthmatic symptoms at 3 months post-discharge. More than 70% of the pH1N1-infected children without a pre-infection diagnosis of asthma had a negative response on the MCT. None of these children had recurrent wheezing or asthma during the three years following pH1N1 infection. The clinical data demonstrate that pH1N1 infection directly induced transient asthma-like symptoms in patients regardless of medical history. To elucidate the underlying mechanisms of AHR after pH1N1 infection in mice, wild-type and $Rag1^{-/-}$ mice that lack T and B cells were adapted. pH1N1 infection effectively induces lung inflammation including cell infiltration and inflammatory cytokine productions in mice. It also induces significantly increased AHR and associated with an elevation in IL-33 level and increased numbers and activity of innate lymphoid cell 2 (ILC2). Small amount of Th2 type cytokines such as IL-4, IL-5, and IL-10 were detected in BALF after pH1N1 infection. The current study demonstrates that pH1N1 infection can stimulate the rapid development of AHR and Th2-type cytokine secretion in mice via the

activation of ILC2; this response may be activated independently from adaptive immunity.

The second part of this study shows that development and characterization of indirect ELISA system using recombinant HA1 protein of canine influenza virus (CIV) H3N2 to apply for the potential use of serosurveillance against virus in dog. CIV H3N2-specific indirect ELISA was established using recombinant HA1 protein (baculovirus-expression system) as a coating antigen. A total 65 CIV H3N2-positive or negative canine sera were tested by the indirect ELISA for receiver operating characteristic (ROC) analysis and compared with hemagglutination inhibition (HI) test. The archived canine sera were collected 10 days after intranasal inoculation of canine H3N2, seasonal H3N2 (A/Brisbane/10/2007) and pandemic H1N1 influenza virus (A/California/04/2009), respectively, were used for the cross-reaction test. Adjusted OD of 0.36 was determined to be an optimal cut-off value for seropositivity. Also, indirect ELISA showed 93.5% and 94.7% of sensitivity and specificity, respectively, when compared to HI test. Cross-reaction test was also performed using the archive canine sera related to CIV H3N2, seasonal H3N2 (human) and pandemic H1N1 (human) influenza viruses. Based on the data in this study, the HA1 of CIV H3N2-specific indirect ELISA can be used to survey the canine H3N2-specific IgG in large scale with simple procedure of which result is comparable with HI test.

Taken together, these results suggest that influenza infection can trigger AHR both in human and mice. And for the diagnosis and serosurveillance for influenza infection and exposure, indirect-ELISA system can support the

establishment of various subtypes of influenza virus HA1 protein-producing pipeline as a promising and useful tool targeting influenza virus outbreaks in human beings.

Keywords: innate immune response, influenza A H1N1 virus, airway hyperresponsiveness, innate lymphoid cells, ILC2, type 2 immunity, acute asthma, canine influenza, H3N2, indirect ELISA, HA1 protein

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Liat of Abbreviations

ACRONYM	FULL NAME
AHR	Airway hyperresponsiveness
aMP	Alveolar macrophage
APC	Allophycocyanin
APCs	Antigen-presenting cells
ATI	Type I alveolar epithelial cells
ATII	Type II alveolar epithelial cells
AUC	Area under curve
BALF	Bronchoalveolar larvage fluid
CBA	Cytokine bead assay
CIV	Canine influenza virus
cvRNA	Complementary viral RNA
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
d.p.i.	Days post infection
ELISA	Enzyme-linked immunosorbant assay
FALC	Fat-associated lymphoid cluster
FEV1	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate
GATA3	GATA binding protein 3
HA	Hemagglutinin
HI	Hemagglutination inhibition
HPAI	Highly pathogenic avian influenza
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cells
IRF	Interferon regulatory factor
Lin	Lineage
Lti	Lymphoid tissue-inducer

M1	Matrix protein 1
M2	Matrix protein 2
MCT	Methacholine challenge test
MDA-5	Melanoma differentiation-associated protein 5
MNT	Microneutralization test
m.o.i.	Multiplicity of infection
NA	Neuraminidase
NK cells	Natural killer cells
NKT cells	Natural killer T cells
NLRP3	NOD-like receptor family pyrin domain containing 3
NOD	Nucleotide oligomerization domain
NP	Nucleocapsid protein
NS	Nonstructural protein
nt	Nucleotide
OIE	Office International des Epizooties (World Organization for Animal Health)
OVA	Ovalbumin
PAMP	Pattern-associated molecular pattern
PC20	20% fall in FEV1
PE	Phycoerythrin
PMN	Polymorphonuclear
PRR	Pattern recognition receptor
Rag1	Recombination activating gene 1
RIG-I	Retinoic-acid inducible protein I
R_L	Lung resistance
RNP	Ribonucleoprotein
ROC	Receiver operating characteristic
RORγt	Retinoic-acid receptor-related orphan receptor γt
RSV	Respiratory syncytial virus
SA	Sialic acid
STAT1	Signal transducer and activators of transcription 1

TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TMB	Tetra-methylbenzidine
vRNA	Viral RNA
WT	Wild-type

Chapter I. Literature Review

I. Influenza Virus

1. Introduction

Influenza caused by influenza virus infection is an acute respiratory inflammation and a serious respiratory illness in humans (1, 2). It has been responsible for millions of deaths worldwide. The influenza virus was first isolated in 1932 in the laboratory by Smith, Andrew, and Laidlaw; however, the history of influenza infection is much earlier than its discovery (3). According to historical records, Hippocrates recorded cases of virus infection, the most early influenza, in early Greek writings of 430 B.C. (4). Scientists collected similar clinical syndromes propagating in a given period at a given location (5). In the 15th centry people called it “influenza”, which comes from the Italian word “influence” because this illness is influenced by unfavorable astrological effect (6). Scientifically reliable references were described in the late 1880s that suggested the influenza virus subtypes, such as H2 and H3, according to sera collected from individuals living in that period (3). History of the influenza pandemic is illustrated in Figure 1-1. Outbreaks constantly occurred in 15 to 40 year interval (3, 4). Recently, there has been a great increase of understanding about the characteristics, period of waves, symptoms, and infection mechanisms of influenza virus than before but things still remain unclear and needs further understanding. In this chapter, I will briefly review current knowledge of influenza virus and innate immune responses related to influenza virus infection.

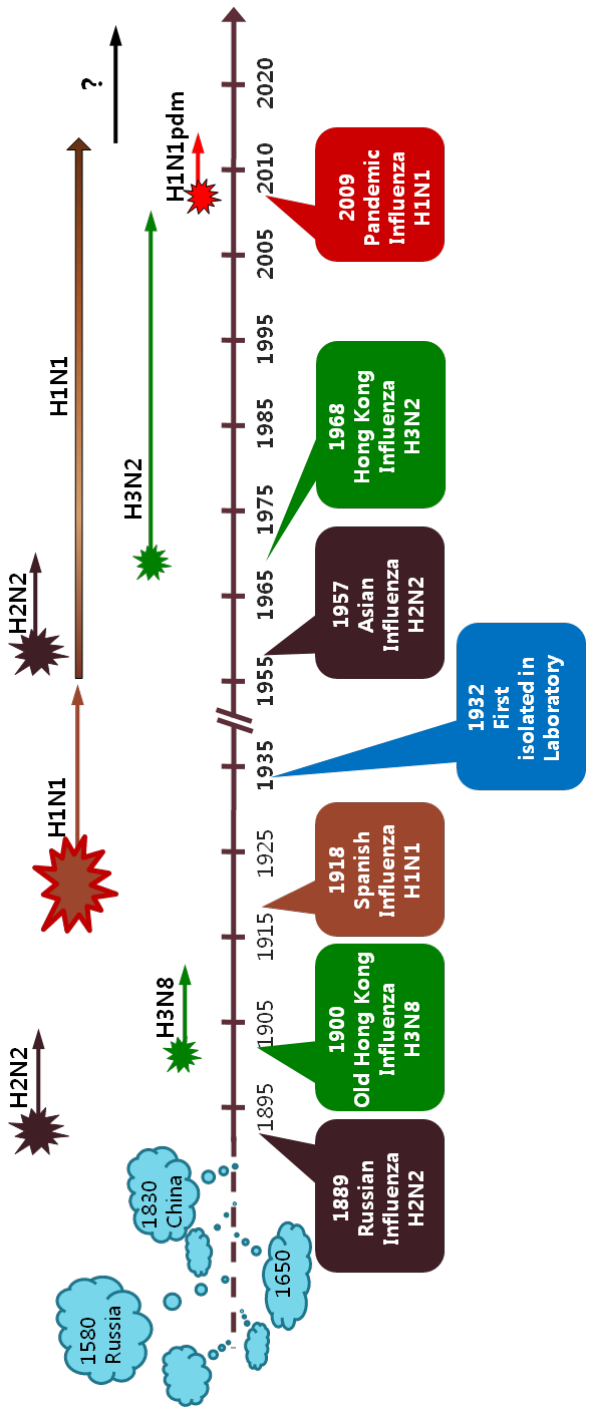

Figure 1-1. History of Influenza.

2. **Characteristics of Influenza Virus**

Influenza virus is helical shaped, segmented, single-stranded, negative RNA virus of the *orthomyxovirus* family (Fig. 1-2) (1, 2). Three immunologic types of influenza virus are known, designated A, B, and C. Among them, influenza A virus is the best-studied so far, for its high variation rate and wide host range from aquatic birds to humans (7). Influenza B virus shows less frequent antigenic changes (8). Influenza C virus is antigenically stable, and it can cause mild illness in humans and animals (pigs and dogs) (9).

Influenza A virus genome is comprised of 8 genes, encoding 9 different proteins and 2 nonstructural protein (Table 1-1). The three largest viral RNA (vRNA) segments encode the three polymerase proteins: PB2 (segment 1) (6, 10), PB1 (segment 2) (6, 7, 10), and PA (segment 3) (6, 10). Segment 2 also encodes another protein PB1-F2, which enhances apoptosis of host immune cells, and it is truncated in the 2009 pandemic H1N1 (6, 10). Segments 4, 5, and 6 each encode a single protein: hemagglutinin (HA) (7), nucleocapsid protein (NP) (11), and neuraminidase (NA) (7), respectively. Segment 7 unspliced mRNA encodes the matrix protein (M1) (6, 7, 10), which underlies the virion lipid membrane, while spliced mRNA encodes the ion channel protein (M2), which plays an important role for uncoating of virus in the endosome (6, 7, 10). Segment 8 also has unspliced mRNA for nonstructural protein (NS1), which has inhibition activity to antiviral response in host cells (6, 10), and spliced mRNA for nonstructural protein (NS2), which is vRNP nuclear export protein (6, 10).

Table 1-1. Influenza A virus genome RNAs and proteins.

RNA segment	*Protein encoded*	*Protein function*
1	PB2	Part of RNA transcriptase components; cellular pre-mRNA cap recognition and binding
2	PB1	RNA-dependent RNA polymerase
	PB1-F2	Localized in mitochondria; proapoptotic activity
3	PA	Part of RNA transcriptase components; endonuclease (implicated in cap snatching)
4	HA	Hemagglutinin; trimer; involved in attachment to sialic acid in host cells
5	NP	Neucleocapsid protein; protect viral RNA from nuclease
6	NA	Neuraminidase; tetramer; involved in cleavage of neuraminic acid from the cell surface
7	M1	Matrix protein, vRNP unclear export, and virus budding
	M2	Integral membrane protein; ion channel
8	NS1	Nonstructual protein; inhibition of antiviral response in host cells
	NS2	Nonstructual protein; vRNP nuclear export

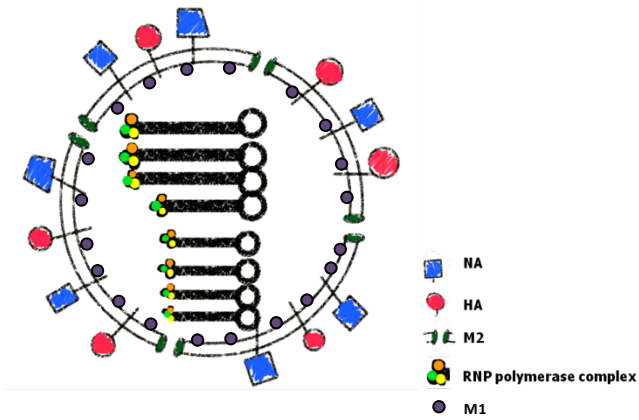

Figure 1-2. Schematic diagram of influenza A virus virion.

Influenza virus envelope is comprised of a lipid bilyayer containing HA, NA, and M2, which are presented on their surface, and M1 underneath the membrane (Fig. 1-2). HA and NA are the main antigenic determinant of influenza A virus. So far, 18 subtypes of HA (H1-H18) and 11 subtypes of NA (N1-N11) were isolated (12, 13).

3. Antigenic Drift and Antigenic Shift

Combination and recombination of those two surface glycoproteins, HA and NA, brings new strains of influenza A virus. The feature of high rate of recombination between strains comes from the discrete segment of viral genome (7). The combination of two proteins (HA and NA) by gene reassortment arises from co-infected single host such as swine which served as a ‘mixing vessel’ in 2009 (14). It is called “antigenic shift” and is the main reason for influenza pandemics, usually. Despite this big change, every year, influenza virus undergoes “antigenic drift” process, substituting one or more amino acid without affecting the HA framework (15). Recently, it was revealed that the 1918 pandemic was not caused by gene reassortment but direct point mutation; accumulated antigenic drift of the avian influenza (16). So far, it is believed that the birth of the 1918 pandemic virus was the result of gene reassortment (6).

4. Life Cycle of Influenza Virus

Entry of the influenza virus as well as immune responses against it started from the binding of HA on the virus surface to sialic acid (SA)-containing glycoproteins and glycolipids on host epithelial cell surface in the respiratory

tract (17). HA from the avian influenza virus binds to α2,3-Gal, and the human virus binds to α2,6-Gal linked SA; the affinity of this bond is important to determine tissue and host tropisms of the virus as well as influenza pathogenesis (18). Human influenza A viruses attach to the upper respiratory tract such as nose, trachea, or bronchi, where SAα-2,6Gal are mainly expressed (19, 20). However, preferential target of a highly pathogenic avian influenza (HPAI) H5N1 is the lower respiratory tract, which expresses an abundance of SAα-2,3Gal, therefore, human-to-human transmission is relatively rare than other strains of influenza virus (20). After HA binds to this SA, influenza viruses is primed for endocytosis and reach to the perinuclear region. During this step, HA is cleaved into HA1 and HA2 by conformational change in low pH (between pH 5 and pH 6) of endosome (21), and this process promotes activation of membrane fusion between the virus and host cells, leading to the release of the ribonucleoproteins (RNPs) into the cytoplasm (uncoating). Then, import of the viral genome into the nucleus occurs (22).

The translocated viral RNP moving into the host nucleus starts transcription and replication using their RNA-dependent RNA polymerase. The original single stranded, negative sense vRNA segments served as templates for the transcription of viral mRNAs and full-length complementary vRNAs (cvRNA) (7, 23). Transcribed mRNAs produce viral proteins essential for replication of cvRNA/vRNA. The transcription and replication step is regulated by virus-derived 22-27 nt length, small viral RNA (23).

Since complete virions of influenza virus are not present within the host cells, they should be released from the cells undergoing continuous process of assembly and budding. vRNP is synthesized within the nucleus and exported into the cytoplasm, then, transported to the apical plasma membrane (24, 25). vRNP segments do not reach the plasma membrane individually. They grouped (2~4 segments in a group; subcomplex) and were exported from the nucleus, then, they underwent further assembly *en route* to the plasma membrane from the cytoplasm via dynamic colonization events (25). Influenza virus budding is a complicated event, which requires tightly organized assembly of multiple different viral proteins and genes. The viral transmembrane proteins, HA and NA, use cellular exocytic transport pathway and process the determinants for lipid raft association and apical transport in their transmembrane domain (24). HA and NA initiate budding event; clustered vRNPs move to HA and NA accumulated plasma membrane and sequentially, M1 and M2 are further processed and completion of this event by releasing virus particle from the host cells is the cleavage of sialic acid linkage by NA (26, 27). Budding forms of influenza A virus show two shapes, usually clinically isolated viruses show long filamentous particles, while laboratory-adapted strains are predominantly spherical (28). However, it was discovered that spherical from is more efficient for the human-to-human transmission than filamentous form (26, 28).

5. Recognition and Antiviral Responses of Host Cells against Influenza Virus Infection

The primary target cells of human influenza viruses are the epithelial cells or rarely alveolar macrophages (aMPs). They predominantly attach to the surface of ciliated epithelial cells, occasionally attach to goblet cells, and rarely attach to brochiolar noncilliated cuboidal epithelial cells (19, 29). In these sites of infection, influenza virus starts to multiply within the host cells, especially epithelial cells, but monocytes/macrophages and other leukocytes are also infected (30). Epithelial cells, aMPs, neutrophils and DCs recognize pattern-associated molecular patterns (PAMPs) of influenza virus or virus particle by pattern recognition receptor (PRR), which is expressed inside and outside of cells (31) (see table 1-2 for detail). It initiates antiviral signal cascades, resulting in the production of interferons (IFNs), cytokines and chemokines (32). Influenza virus is recognized by host immune system via PRR such as Toll-like receptors (TLR3, TLR7, and TLR8), retinoic-acid-inducible protein I (RIG-I) and nucleotide oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) existed in endosome and cytosol (table 1-2) (33). RIG-I and NLRP3 detect virus in the cytosol of infected cells, while TLR3 and TLR7/8 detect virus-infected cells and viral RNA in the endosome. Then, antiviral immunity is initiated by producing type I IFN and proinflammatory cytokines such as IL-6, IL-12, TNF-α, IL-8, IP-10, RANTES, MIP-1β, and subsequently, robust adaptive immune response (34, 35).

Table 1-2. Host PRR response to virus PAMP

Host factor	*Viral factor*	*Host response*
TLR TLR3	Viral dsRNA	Type I IFN production
		NFκB activation
TLR7/8	Viral ssRNA	Type I IFN production
		NFκB activation
RIG-I	5'-ppp viral ssRNA	Type I IFN production
		NFκB activation
MDA-5	dsRNA	Type I IFN production
NLRP3	Influenza RNPs	Caspase I activation

6. Complication of Influenza Virus Infection

Several influenza A virus subtypes, including 1918 (H1N1), 1968 (H3N2), 2009 (H1N1), and most recently 2013 (H7N9), became a pandemic and led many people to be sick. About 20% of children and 5% of adults worldwide develop symptomatic influenza A and B each year (2). Patients show combined features from the common cold symptoms to acute onset of headaches, muscle and body aches, and malaise, and so forth (36, 37). Conjunctivitis caused by influenza virus infection is one of the unique features (38). In the case of H7N7, four people developed purulent conjunctivitis as they directly contacted animal sneeze to their face in 1980 in the USA, and a woman who kept duck showed conjunctivitis and was predicted that she was directly infected with the avian influenza in 1996 in UK (38). In 1997, several cases of the influenza H5N1 infected patients suffered from acute respiratory distress syndrome or multiple organ failure,

and 30% of them died, even though most were previously healthy young adults (39). The reason for this viral complication or unexpected various symptoms is the consequence of emergence of unpredictable influenza virus variation that may come from major or minor mutations of genes in influenza virus through antigenic drift and shift. Early treatment against influenza infection in human is crucial since there is no known effective antiviral agent after the release of the virus. As antibiotics were not available in the 1918 pandemic, secondary bacterial pneumonia was a major cause of death among those infected with the virus (40). Most popular antiviral reagent is oseltamivir (Tamiflu®), which is a neuraminidase inhibitor. It blocks the virus release from infected cells. Therefore, early drug treatment is critical issue for controlling the spreading of the virus considering the antiviral mechanisms of medicine (41).

7. Influenza Vaccine

All persons, 6 months and older, should be vaccinated annually because of the antigen drift and shift of influenza virus. Anyone can get flu, however, half of the estimated influenza virus infected people do not develop clinical symptoms; they are only important carriers for spreading of the virus (36). However, young children, people 65 and older, pregnant women, and people with certain health conditions such as immunocompromised, kidney disease, or nervous system disorder get worser cases of the flu than others (42). Flu vaccine shows to be the most effective protection against flu infection and its complications. Current vaccine formulations target influenza A virus (H1N1

and H3N2), and influenza B virus, which are frequently isolated from human recently (www.cdc.go.kr) (43). Most conventional vaccine is an inactivated vaccine, which is produced in embyonated chicken egg. It is given intramuscularly by injection as a “flu shot”. It induces virus neutralizing antibody, and adjuvant, MF59 and AS03, are used to improve their immunogenicity (44). Live attenuated influenza vaccine is sprayed into the nostrils. It induces site-direct mucosal antibody responses and cytotoxic T lymphocytes (CTL) responses, following uptake and processing by antigen-presenting cells (APCs) (43). Egg-based influenza vaccine has many disadvantages such as time consuming, high cost, limit of raw materials, egg allergy, and so forth. It is difficult to supply enough vaccine to all human beings.

8. Serological Diagnosis Tools for Influenza Virus Neutralizing Antibody

It is important to know the vaccine efficacy after vaccination as well as the diagnosis of virus infection. Serological tests to detect specific antibodies from influenza infection or influenza vaccination in human are critical for disease prevention, preparation for control program, and vaccine savings. There are three available serological tests including; hemagglutination inhibition (HI) test, microneutralization test (MNT), and enzyme-linked immunosorbent assay (ELISA) (45, 46). HI test is technically simple but limited in detecting antibody and unable to recognize antibody subtypes. Moreover, it requires fresh erythrocytes for each experimental run. MNT is sensitive, but it is time-consuming, has a labor-intensive process, and requires

many facilities (47). Both HI and MNT use live virus to be performed, so it needs biocontainment facilities and experts to reduce the exposure of many dangerous situations during handling of the virus. Anti-influenza HA antibody directly correlates to protection ability (48). ELISA rapidly detects anti-influenza-specific antibody rather than living virus in the sample (Fig. 1-3) within 5 hrs to 1 day, so it is relatively easier than MNT and more sensitive than HI. Moreover, ELISA system can determine antibody isotypes and subtypes with simple manipulation of protocol and a small amount of samples. Since ELISA does not require the use of viral particles, it can be easily and quickly implemented in any basic laboratory at hospitals, and large-scale tests can be conducted to determine the prevalence of disease and vaccine efficacy.

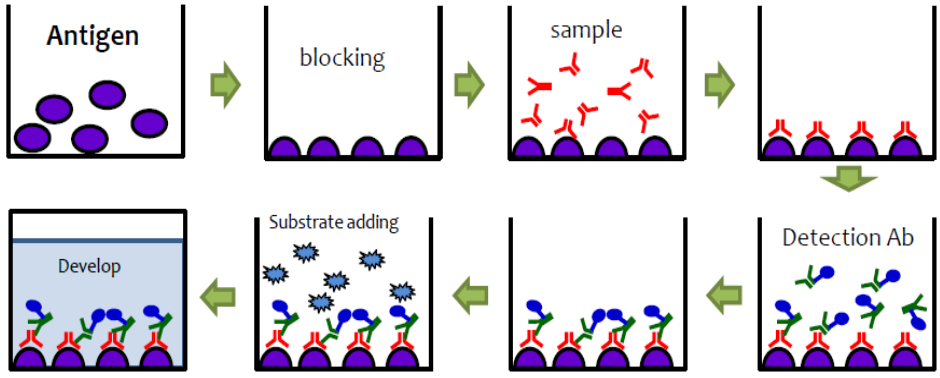

Figure 1-3. Procedures of antigen-specific indirect ELISA.

II. Innate Immune Systems in Host Defense Mechanisms

1. Innate Immune Systems in Mucosal Sites

The human body as well as other living organisms have a two-parts of barrieres; skin for the outside and mucosal lining for the inside. These barriers are separate outside and inside physically (ex; rhythmic movement in the ascending direction) and chemically (ex; secretion of antimicrobial peptide), and they have their own specialized immune system. As the first line of defense, excluding the skin barrier, the innate immune system is very important in protecting our body. Various pathogens and living agents, including allergen or disease causing agents, interact with the innate immune system daily inside and outside of the body. They are detected and destroyed within a couple of minutes to days by innate immune system. Before beginning of the inflammation, evolutionarily conserved defense mechanism of eukaryotic cells is processed against pathogens (49, 50). Innate immune system provides initial protection against microorganisms and stimulates the adaptive immune response (51). Moreover, it refers to all aspect of protection against infection that are genetically predetermined and do not require prior exposure to the foreign potential pathogens (50). We inhale 10,000 liters of air per day. Lung is exposed to a large number of air-bone pathogens as a result of these inhalations (51).

2. Airway Epithelial Cell Lining

In the mucosal region, epithelial cells not only provide a passive barrier function, but also actively contribute to the innate immune system. Epithelial cell layer is tightly connected to tight junctions, adherens junctions and desmosomes, and these provide physical strength and cell-to-cell communication (52). Epithelium is comprised of several types of cells: ciliated cells, mucous secretion cells, and poorly-differentiated cells, however, the cell types of airway and intestinal epithelium are slightly different. Table 3 shows types of epithelial cells in the respiratory site.

The airway epithelium is in a pseudo-stratified form, and there are at least eight morphological distinct cell types, although the function is classified into only three categories: basal, ciliated and secretory (53). Over 50% of epithelial cell type is cilated epithelial cells. They are terminally differentiated from the clara cells (Club cells; bronchiolar exocrine cells) or basal cells, and their main function is directional transport of mucus from lung to throat. Goblet cells produce mucus, and mucus traps foreign particles, which is cleared out by the movement of ciliated cells. Clara cells secrete mature surfactant proteins A, B, D and several detoxifying enzymes. Moreover, they are putative progenitor cells along with basal cells and differentiate into ciliated cells, and they are capable of self renewing when tissues become injured under specific conditions (54, 55). In the alveoli, 90% of peripheral lung surface is covered by alveolar epithelial cells type I (ATI), which mainly play a roles in gas exchange. Type II alveolar epithelial cells (ATII) are precursors of ATI, moreover, these cells secrete surfactant proteins

which are involved in innate immune defense and antimicrobial proteins such as lysozyme, complement components (e.g., C2, C3, C4, and C5) as well as a variety of cytokines and chemokines (56, 57). These cells are main source of secreted IL-33, which is important in allergy and lung inflammation and has anti-inflammatory roles (55, 58). Neuroendocrine cells may play a role in regulation of epithelial cell proliferation and differentiation (54). As most of the mature lung is non-proliferative; resident cells survive prolonged except in inflammatory conditions by pathogen infection and allergen exposure. In certain conditions of inflammation, an injury site is quickly closed and replaced with newly differentiated cells. ATII, clara cells, basal cells and neuroendicrine cells have important roles in this repair (54, 55).

Table 1-3. Type of epithelial cells in the lung.

Airway epithelium	*Role*
Alveolar epithelial cells I (ATI)	Gas exchange
Alveolar epithelial cells II (ATII)	Progenitor cells, secreting cells; surfactant, cytokines
Clara cells (Club cells)	Secreting cells; surfactant
Goblet cells	Mucus secretion
Typical basal cells	Progenitor cells
Neuroendocrine cells	Regulate cell proliferation and differentiation

3. Macrophages in the Lung

Macrophages are classically known for professional phagocytes that remove and clear the dying cells and foreign antigens both in the steady state conditions and inflammatory conditions. Macrophage has diverse functions and names according to the tissue where they are located. In the lung, there are alveolar macrophages (aMPs) and interstitial macrophages. aMPs exist in the airspace and are the first cells of the immune systems to encounter inhaled allergens and airborne pathogens. In normal condition, aMPs are a small population in airspace. Murine aMPs poorly present antigens to the T cells, instead of that they rapidly transport antigens to secondary lymphoid tissue in lung in early time of infection (59). Human aMPs also have poor antigen presentation ability to the T cells and lack co-stimulatory molecules B7-1 (CD80), B7-2 (CD86) despite the condition of stimulation with IFN-γ (60). They showed decreased phagocytic activity compared to interstitial macrophage (61). Both aMPs from mice and human are known to contribute to maintaining tolerance of lung by producing immunosuppressive molecules such as prostaglandin and TGF-β, and moreover, aMP-secreting retinoic acid and TGF-β have effects on the foxp3 expression in naive $CD4^+$ T cells to proliferate regulatory T cells (61, 62). In an inflammatory condition, macrophages are recruited to the airway and become aMPs. They can be divided into two groups: classically activated M1 macrophage or alternatively activated M2 macrophage. The concept of classification is similar to Th1 and Th2 cells, i.e., IFN-γ in cell-mediated immunity to intracellular infection for the former and th2 type cytokines such as IL-4 in extracellular parasitic

infection or allergen response for the latter is analogous with M1 and M2 macrophage, respectively (63). M2 cell populations are increased in inflammatory lung diseases such as allergy induced inflammation and the idiopathic pulmonary fibrosis, which is a devastating disease (61, 64). In the virus infection, classical M1 macrophages are activated since the main stimuli of these cell population is IFN-γ, then activate STAT1 (signal transducer and activators of transcription 1) and IRF (interferon regulatory factors) signaling (63). aMPs are important for host survival against virus infection such as influenza virus and vaccinia virus infection. In the absence of aMPs, mice resulted in the loss of lung function with severe morbidity after influenza infection. Defect of aMPs is succeptable for the other infection such as secondary bacterial infection called bacterial superinfection. After the influenza infection, influenza virus impedes the phagocytic receptors and phagocytosis of aMPs (65). For the protective roles of aMPs, GM-CSF treatment improves the survival from influenza infection, increased numbers of aMPs and increased resistance to apoptotic effects of influenza virus infection (66, 67). In vivo, the main source of GM-CSF is alveolar epithelial cells, and it is important to stimulate aMPs and to infiltrate other immune cells such as monocytes and dendritic cells (DCs) (67). Moreover, aMPs secrete various cytokines and chemokines upon influenza infection, especially type I IFNs which are critical for antiviral response (68). So the harmony of the epithelial cells and immune cells resident in the lung, such as aMPs, are important to maintain homeostasis and to protect pathogenic infection.

4. Other Immune Cells

DC is also important for host defense even aMP is fascinating in the lung. Originally, it is known that conventional DCs rapidly differentiate to myeloid DCs after viral infection, and they detect viral antigen in early time of infection and transfer it into the draining lymph nodes to initiate antiviral response (69). Plasmacytoid DC is a small population in the steady state condition but they are rapidly recruited and become a predominant population in the lung during infection and one of a major source of IFN-α (69). They have a role as a linker between innate immunity and adaptive immunity in the lung.

Neutrophils are usually the earliest recruited immune cells after pathogenic infection in the body including influenza virus infection in the lung. Excessive numbers of neutrophils are immunopathogenic (neutrophils producing oxidants is injurious) in the early time of infection, however, they usually have protective roles against influenza infection including viral clearance and induction of innate immune response. Neutrophils uptake influenza virus and serve the viral antigen to anti-viral effector $CD8^+$ T cells (70).

Natural killer (NK) cells are small fraction of lymphocytes, and they are a critical source of IFNs and contribute in coltrolling virus replication. The NK cell producing IL-15 influence the proliferation and recruitment of $CD8^+$ T cells and NK cells into the site of inflammation (71). IL-22, produced by NK and NKT cells, has an effect on repairing the epithelial damage caused by influenza infection (67). NK cells also improve the function of γδT cells. γδT cells produce IL-17, and this cytokine is an important trigger of neutrophils

recruitment and starts the inflammation, consequently leading to adaptive immune responses (67).

5. Innate Lymphoid Cells

Innate lymphoid cells (ILCs) are recently characterized and identified by many immunologists for their important roles in innate immunity, regulation of tissue homeostasis and inflammation. ILCs secrete Th cell-associated cytokines, but they do not express cell surface markers, which is expressed on other immune cells. NK cells and lymphoid tissue-inducer (Lti) cells are also comprised in the ILCs (72, 73). ILCs are originated from a common lymphoid progenitor of a fetal liver or adult bone marrow (74). All ILCs require the transcriptional repressor Id2 and IL-7 signaling for development and survival of themselves. These ILCs are categorized into three groups based on the cytokines they produce. For examples, cell that produce Th-1 type cytokines such as IFN-γ, and TNF-α are grouped as ILC1, Th-2 type cytokine producing cells such as IL-4, IL-5, and IL-13 are grouped as ILC2, and group 3 ILCs produce both Th-1 and Th2 type cytokines as well as Th-17 associated cytokines (73). Group 1 ILCs are similar to NK cells; NK cells, which mainly produce IFN-γ but they are weakly cytotoxic, are also included in this group. Group 2 ILCs secrete Th-2 cytokines and explained below in detail. Group 3 ILCs are comprised of LTi cells and ILC3 (Fig. 1-4).

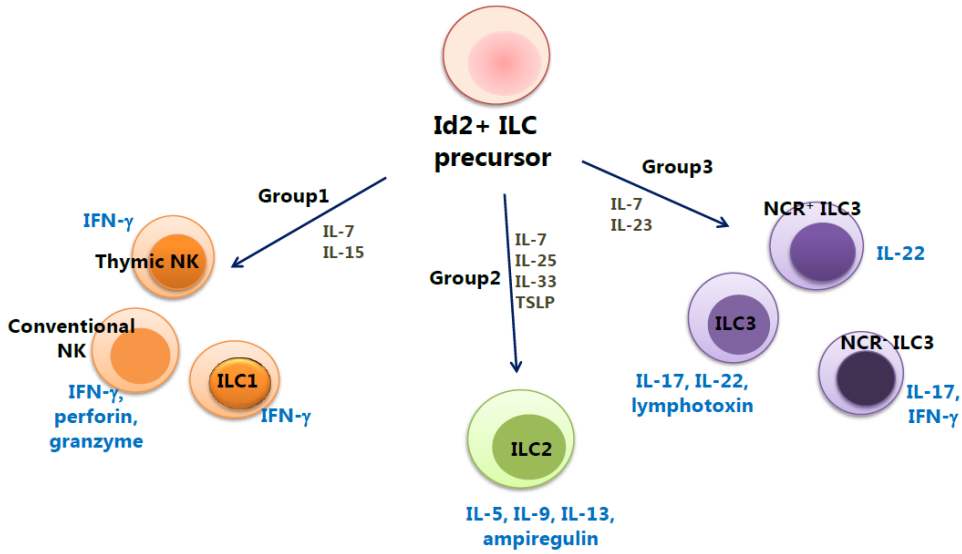

Figure 1-4. ILC subsets and the cytokines it produces. Three groups of ILC have common progenitor cells, Id2-dependent lymphoid progenitor cells. Various cytokines are influenced on ILC to differentiate into ILC1, ILC2, and ILC3.

Table 1-4. Phenotypic markers of ILC subsets.

ILC lineage	Common features	Mouse	Human
ILC1	Lin^- (mouse; CD11c, CD11b, CD3, CD4, CD19, CD49b, NK1.1, GR-1, F4/80, FcεR1, B220, TCRβ, Ter119) (human; CD1a, CD3, CD11c, CD34, CD123, TCRαβ, TCRγδ, BDCA2, FcεRI, CD19, CD14, CD16)	$CD127^+IL1R^+$ $IL\text{-}12Rb2^+$	$CD56^+NKp46^+$ $NKp30^+NKp44^+$ $IL\text{-}7R^-$
ILC2		$ST2^+sca\text{-}1^+$ $IL\text{-}7a^+Icos^+$	$IL\text{-}7Ra^+CD161^+$ $CRTH2^+$
ILC3		$RORrt^+NKp46^+$ $IL\text{-}7Ra^{int}$	$CD56^+NKp46^+$ $NKp30^+NKp44^+$ $IL\text{-}7Ra^+$

ILC, innate lymphoid cell; Lin, lineage marker; NK, natural killer; RORγt, retinoic acid receptor-related orphan receptor-γt; TCR, T cell receptor.

Interestingly, these cells do not express T cell receptor (TCR), and thus, do not respond in an antigen specific manner (73). The specific markers for these cells are studied by many research groups. Even the specific surface markers are slightly different between ILCs, however, they typically do not express lineage cell markers such as CD11c, CD11b, CD3, CD19, CD49b, NK1.1, GR-1, F4/80 (75, 76). Details of surface markers in ILCs are summarized in Table 1-4.

6. Group 2 ILC

The group 2 ILC (ILC2) consists of heterogeneous cell population, and it is discovered by different research groups, so it is called different names: nuocytes, natural helper cells, innate type 2 helper cells, and type 2 innate lymphoid cells; multi-potent progenitor type 2 cells also consist of ILC2, but they have the potential to further differentiate (75, 77-80). Now those populations are called ILC2, because they secrete type 2 cytokines such as IL-5, IL-9, IL-13, and a small amount of IL-4. They mainly exist in mucosal regions such as the intestine and fat-associated lymphoid cluster (FALC) in the mesentery, however, they are also found in the spleen, liver, and bone marrow as a small population (75, 78). They even showed different surface markers according to their activating status and isolated tissue, however, they generally do not express lineage surface markers expressed on immune cells such as T cells, NK cells, B cells, eosinophils, neutrophils, basophils, and mast cells (Table. 1-4) (78). ILC2 expresses CD45, c-Kit, Sca-1, T1/ST2 (IL-33R), and IL-17Rb (IL-25R), as a surface marker and transcript factor GATA3 (75, 81). ILC2 produces IL-13 in response to epithelial cytokines IL-25 and IL-33 and expand after helminth infection in intestine and have a protective role even in the absence of adaptive immunity (78). Also, FALC-associated ILC2 is important for homeostasis. For example, it supports the self-renewal of B1 cells and enhances IgA production of these cells (75).

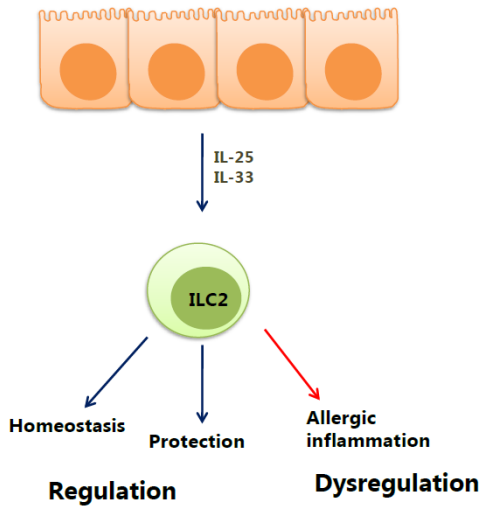

Figure 1-5. ILC2 has two faces of biological roles.

ILC2 existing in the lung has diverse effects on the tissue homeostasis and airway allergic response, but the role of ILC2 in the lung is controversial (Fig. 1-5). Monticelli *et al* reported that ILC2 is expanded in response to H1N1 influenza virus infection. However, these cells did not affect viral load, but they are important in maintaining the integrity of airway epithelial cell barrier and restoring tissue homeostasis after viral infection (76). Another study showed that ILC2 producing type 2 cytokines affects the pathogenic and airway hyperreactiveness (AHR; cardinal feature of asthma) after H3N1 infection in the absence of adaptive immunity (82). It is unclear why the ILC2 shows different features against influenza infection, however, a study indicated that it may be caused by the different subtypes of viruses used in each study (83).

7. ILC2 and Asthma

Dyregulation of cytokine secretion by ILC2 can cause tissue damage and pathologic inflammation in lung (Fig. 1-5). Airway inflammation and pathology, characterized with type 2 cytokines secretion level, allergen sensitization and adaptive immunity, may result in allergy and asthma. Asthma can also be induced by non-allergen agents such as viral infection, obesity, or air pollution: ozone, cigarette smoke, and diesel particles (84). It is often associated with neutrophils infiltration in the airway and innate immune response independent of Th2 cells (85). ILC2 secreted type 2 cytokines in responses to IL-25 and IL-33 are also considered for inducing allergic asthma responses in specific conditions. *IL-33* has been identified as an asthma-

related gene (86, 87). IL-33 is produced by variety of cells such as lung stromal cells, airway epithelial cells, airway smooth muscle cells, and alveolar macrophages (58, 88). It is shown to induce production of type 2 cytokines and also contribute to the early- and late-phase allergic rhinitis (89). Anti-IL-33 antibody treatment inhibit OVA-induced airway inflammation by reducing type 2 cytokine secretion and serum IgE production in allergic asthma animal model (90, 91). The study that IL-33R (ST2) deficient mice failed to develop influenza-induced AHR explains the important role of IL-33 in developing AHR (82). IL-33 binds to the T1/ST2, which is a surface marker of ILC2, then effect cell activation (Fig. 1-6) and production of cytokines such as IL-5, IL-9 and IL-13 (Fig. 1-7). ILC2 producing IL-13 also has significant effects on the induction of AHR, especially in the absence of adaptive immunity, thus, the innate cell producing IL-13 is a key factor for inducing AHR (58).

Collectively, ILC2s are critical sources of type 2 cytokines in early time of inflammation induced by either allergen or viral antigen, and this response can be developed regardless of adaptive immunity, especially T cell-immunity.

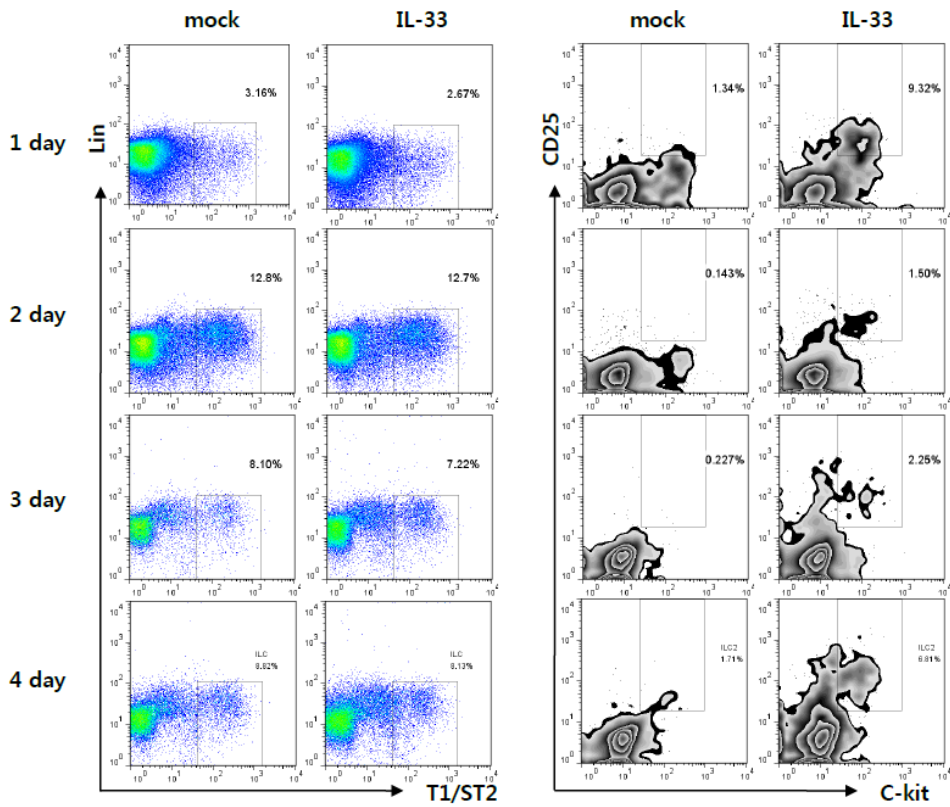

Figure 1-6. Effect of IL-33 on the ILC2 activation. Total lymphoid cells were isolated from the lung of mice and lineage negative populations (Lin^- cells) were sorted out using cell sorter Aria II. Lin^- cells were stimulated with 2.5 ng/ml of recombinant IL-33. At the indicated time point, cells were harvested and the surface markers of ILC2 were stained and analyzed with LSRII.

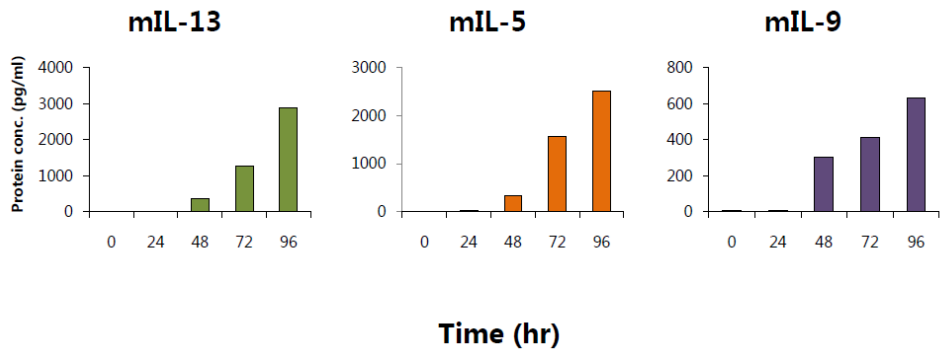

Figure 1-7. IL-33 induces type 2 cytokine secretions in ILC2. Total cells were isolated from lung, and lin$^-$ cells were sorted out. Lin$^-$ cells were stimulated with IL-33 *in vitro*. At the indicated time point, cells were harvested and the cell supernatants were analyzed for cytokine measurement using cytokine bead assay and this measurement analyzed with LSRII.

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Chapter II.

Pandemic Influenza Virus, pH1N1, Induces Asthmatic Symptoms via Activation of Innate Lymphoid Cells

I. Abstract

As a first flu pandemic of 21st century, influenza A/California/04/2009 (pH1N1) was highly pathogenic and lift threatens. Several reports insist that influenza virus infection can cause airway hyperresponsiveness (AHR) in mice. However, there is no report about the relationships between asthmatic symptom and pH1N1 infection both in mice and in patients without previous history of asthma. To quantify asthmatic symptoms in pH1N1-infected children, we administered a methacholine challenge test (MCT) in confirmed pH1N1-infected children who were hospitalized between October 2009 and February 2010 with moderate to severe acute asthmatic symptoms at 3 months post-discharge. More than 70% of the pH1N1-infected children without a pre-infection diagnosis of asthma had a negative response on the MCT. None of these children had recurrent wheezing or asthma during the three years following pH1N1 infection. To elucidate the underlying mechanisms of AHR after pH1N1 infection in a murine model, wild-type and $Rag1^{-/-}$ mice that lack T and B cells were adapted. It effectively induces lung inflammation including cell infiltration and inflammatory cytokines productions. pH1N1 infection induces significantly increased AHR and it was associated with an elevation in IL-33 and increased numbers and activity of innate lymphoid cells 2 (ILC2) levels. Moreover, small amount of Th2 type cytokines such as IL-4, IL-5, and IL-10 were detected in BALF after pH1N1 infection. The current study demonstrates that pH1N1 infection can stimulate the rapid development of AHR and Th2-type cytokine secretion in mice via the

activation of ILC2; this response may be activated independently from adaptive immunity. And the clinical data could be explained that pH1N1 infection directly induced asthmatic symptoms in patients regardless of medical history.

II. Introduction

Asthma, which is a serious chronic lung disease that afflicts approximately 150 million people worldwide (1), is a heterogeneous and genetically complex disease. Asthma is usually induced by an allergen. Allergen-induced Th2 cell activation results in the activation of eosinophils and mast cells, leading to airway hyperplasia. Respiratory viruses such as rhinovirus, respiratory syncytial virus (RSV), and the influenza virus are the most frequent triggers for the allergic exacerbation of asthma (2). Unlike other viruses, the influenza virus induces a Th1 immune response, robust IFN-γ production, consequently activates dendritic cells (DC) via IFN-γ-bolstered Th2-type immunity against recent or sequentially exposed allergens resulting in boosting of allergen response. The influenza virus also induces pulmonary lung inflammation (3).

The influenza virus is comprised of 8 segmented single-stranded negative RNA virus (4, 5). The segmented characteristic of the influenza RNA is what allows the influenza virus to undergo continuous evolutionary antigenic changes during the replication process (6). The emergence of a new strain of the influenza virus can lead to an increase in complications and unexpected symptoms. Several studies have reported that the 2009 pH1N1 virus has been associated with asthma symptoms in a few cases (7-9).

A recent study on the relationships between H3N1 infection and airway hyperresponsiveness (AHR) in the mouse (10) revealed that H3N1 infection activates resident innate lymphoid cells, especially type 2 innate lymphoid

cells (ILC2), via IL-33 secretion, which induces type 2 cytokine secretions and leads to airway hyperplasia. The ILCs represent a heterogeneous group of hematopoietic cells that comprise only a small fraction of the total immune cell population in the epithelial barrier, skin, and lymphoid organ. The roles of the ILCs have been divided into three groups based on their surface markers and cytokine-producing activities (11). ILC2s are the only ILC cell types discovered to date that secrete type 2 cytokines, including IL-5, IL-9, and IL-13 as well as IL-4 in small amounts. ILC2s express the surface markers CD45, IL-7Ra, T/ST2, sca-1, and c-kit, but do not express the lineage cell marker (10-14). ILC2s also express the transcription factor GATA3, which is expressed mainly in Th2 cells.

This study has identified an unprecedented symptom among children diagnosed with the H1N1 virus infection during the 2009 pandemic. These children experienced acute asthmatic symptoms regardless of their prior asthma history. Animal experiments were also performed to determine the underlying mechanisms related to these influenza-induced asthmatic symptoms in the innate immune system, with a particular focus on ILC2 involvement.

III. Materials and Methods

1. Subjects

This study included children under 15 years old who were admitted to the Severance Children's Hospital between October 2009 and February 2010 with respiratory complications resulting from H1N1 influenza infection. pH1N1 infection was confirmed by real-time polymerase chain reaction (PCR) using a nasopharyngeal swab taken when the patients presented with a fever and acute respiratory symptoms such as a cough, rhinorrhea, and/or a sore throat (15). H1N1-infected children with moderate to severe acute asthmatic symptoms were enrolled in the study. Acute asthmatic symptoms were defined as acute episodes of progressively worsening shortness of breath, coughing, wheezing, and chest tightness (16). The initial physical findings of the patients at admission were examined by expert pediatric allergists. The personal and family allergy and asthma history of each patient were collected via a standardized questionnaire (17, 18). Patients performed a methacholine challenge test (MCT) three months post-discharge. Anti-inflammatory drugs and bronchodilators were prohibited one week before the test. The MCT was performed according to the standardized procedure (19). Each patient inhaled increasing concentrations of methacholine (0.075, 0.15, 0.31, 0.62, 1.25, 2.5, 5, 10, 25, and 50 mg/mL) nebulized by a dosimeter (Dosimeter MB3 Mefar, Brescia, Italy) until the forced expiratory volume in 1 second (FEV1) was reduced by 20% from a post-nebulized saline value. AHR was defined as the concentration below 16 mg/mL at which a 20% fall in FEV1 (PC_{20}) was

observed. These patients were reassessed by a telephone interview three years post-discharge. This study was approved by the Institutional Review Board of Severance Hospital (4-2014-0127).

2. Animals

Wild-type (WT) C57BL/6 and C57BL/6 background $Rag1^{-/-}$ mice (8–10 weeks old, weighing 18–20 g) were purchased from Orient-Bio (Seoul, Korea). The animals were housed in animal care facilities in specific pathogen-free conditions at Yonsei University in Seoul, Korea for the duration of the experiments. Sterilized food (Orient-Bio) and water were supplied *ad libitum*. The experiments were approved by the Institutional Animal Care and Use Committee of the Yonsei University College of Medicine.

3. Influenza virus and animal infection

Influenza A virus (A/Seoul/YS-01/2009, pH1N1) was used. Adult female mice (7–8 weeks old) were anesthetized with a zoletil 50 mix (0.015 cc/20 g; VirbacLaboratory, Carros, France) and Rompun injection (0.5 mg/20 g; BayerKorea, Seoul, Korea) and inoculated intranasally with a 1×10^4 plaque-forming unit (pfu) of pH1N1 suspended in 20 μl of PBS. Control mice (mock-infection group) were treated with the same volume of sterilized PBS through the same route.

4. Measurement of AHR in the influenza-infected animal

At 5 days post-infection (dpi), mice were prepped for AHR measurement. Mice were anesthetized, and then were given a tracheotomy, intubated with an intravenous catheter (BD Bioscience; San Jose, CA) and mechanically ventilated using the Flexivent (SCIREQ, Montreal, QC, Canada) according to the manufacturer's instructions. The volume of injection was calculated based on the animal's body weight. Baseline lung resistance and responses at aerosolized PBS were measured, followed by measurements of responses to 0, 3.125, 6.25, 12.5, 25, and 50 mg/ml of serially diluted, aerosolized acetyl-b-methylcholine chloride methacholine (Sigma-Aldrich, St. Louis, MO). Lung resistance was determined as the average of three time measurements at each dose of methacholine per mouse over three independent experiments.

5. Sample collection and measurement of cytokines

Mice were sacrificed immediately following the AHR measurement. To obtain bronchoalveolar lavage fluid (BALF), the lungs of each mouse were cannulated with an intravenous polyethylene catheter (BD Bioscience) and washed three times with 1 ml of sterile PBS. The lungs then were perfused with 10 ml of sterile PBS injected into the right ventricle, then removed and rinsed in sterilized PBS. Whole mouse lungs were homogenated using a disposable homogenizer (BioMasher, Tokyo, Japan) with 1 ml of sterile PBS. Lysates were centrifuged and used for cytokine measurement. Each sample was individually measured for ELISA (eBioscience, San Diego, CA) or cytokine bead assay (CBA; BD Pharmingen, BD Bioscience). In the BALF

samples, TNF-α, IFN-γ and IL-1β were measured by ELISA and MCP-1, RANTES, IL-6, and Th2-type cytokines including IL-4, IL-5, IL-9, IL-10, and IL-13 were measured by a CBA Flex Set kit (BD Bioscience) and analyzed by flow cytometry (LSRII, BD^{TM}). IL-33 was detected in whole lung lysates following a BAL wash.

6. Histopathological analysis

Lungs from randomly selected mock- and H1N1-infected mice were perfused by injecting 10 ml of sterile PBS into the right ventricle, then rinsed with PBS and fixed in 4% formaldehyde overnight at 4°C. The tissues were dehydrated by gradually soaking them in alcohol and xylene. The dehydrated tissues then were embedded in paraffin. The paraffin-embedded specimens were cut into 5 μm sections and stained with hematoxylin and eosin. Disease scores were assessed in a blinded test based on the level of lung tissue destruction, edema, epithelial cell destruction, and polymorphonuclear (PMN) cell infiltration into the inflammation site.

7. Total lymphoid cell isolation from the lung

Whole lungs were removed following perfusion and rinsed with PBS. Lungs were then chopped with scissors, and the tissues were digested using a collagenase IV (CSL4, Worthington Biochemicals, Lakewood Township, NJ; Fraction IX, Sigma-Aldrich) and DNase I (50 U/ml; Sigma-Aldrich) in RPMI1640 medium containing 10% FBS. Samples were incubated in a shaking incubator for 60 min at 37°C. Total lymphocytes were then washed

and enriched by a discontinuous density gradient of Percoll (Amersham Biosciences, Pittsburgh, PA).

8. Flow cytometry

Total isolated cells were first incubated with Fc block antibody (2.4G2; BD pharmingen) and stained with fluorescein isothiocyanate (FITC) conjugated T1/ST2 (DJ8; MD Bioproducts, St Paul, MN), phycoerythrin (PE)-conjugated anti-CD3 (17A2, BD Pharmingen), anti-CD19 (1D3, BD Pharmingen), anti-CD11c (HL3, BD Pharmingen), anti-CD11b (M1/70, BD Pharmingen), anti-CD49b (DX5, Biolegend, San Diego, CA), anti-F4/80 (BM8, eBioscience), anti-B220 (RA3-6B2, BD Pharmingen), anti-FcεR1 (MAR-1, eBioscience); PerCP-Cy5.5 rat-anti-mouse CD25 (PC61, BD Pharmingen), allophycocyanin (APC)-conjugated anti-c-Kit (ACK2, Biolegend, San Diego, CA), AlexaFluor 700-conjugated anti-mouse Ly6A/E (Sca-1, D7, eBioscience), and Alexa Fluor 780 conjugated anti-CD45 (30-F11, BD Pharmingen). Cells were analyzed on an LSRII (BD Bioscience) with Flowjo 7.6.5 software.

9. Real-time PCR

Total RNA was extracted with a TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA), and 5 μg of RNA was converted into cDNA using Superscript III Reverse Transcriptase (Invitrogen Life Technologies). The products were used as a template for each cytokine-specific real-time PCR set for the amplification of GAPDH, IL-4, IL-5, and IL-13. TaqMan

primer-probe sets for each cytokine were purchased from Applied Biosystems online. The amplification reactions were performed with 100–200 ng of cDNA, TaqMan Universal PCR Master Mix (Applied Biosystems), and each of the designated probes (Applied Biosystems). Gene expression quantification was performed using a StepOne real-time PCR system (Applied Biosystems). The levels of mRNA expression were displayed as the expression units of each target gene relative to the expression units of expressed GAPDH.

10. Statistical analysis

For the human clinical data, numerical variables were expressed as the mean ± SD. Numerical parameters with a non-normal distribution (e.g., blood eosinophil count, serum total IgE) were log-transformed before analysis. Subject demographics in the patients with and without a prior history of asthma were compared using two-sample t-tests or Chi-square tests (e.g., age and sex). A p-value < 0.05 was considered statistically significant. The Statistical Package for the Social Sciences software (version 20.0, SPSS Inc., Chicago, IL) was used for all analyses. For the *in vivo* animal experiments, all data were expressed as the mean ± SD. Statistical comparisons between experimental groups were performed using the Student's t-test in GraphPad Prism 5.

IV. Results

1. Epidemiology of pH1N1-infected children

A total of 4,685 children were confirmed with H1N1 infection at Severance Children's Hospital during the study period. A total of 184 (3.9%) of these children were hospitalized due to respiratory complications from H1N1, including acute asthmatic symptoms, pneumonia, croup, and acute tonsillopharyngitis. Among these 184 inpatients, 68 (37%) exhibited H1N1-induced acute asthmatic symptoms. Forty-nine children with acute asthmatic symptoms performed the MCT three months post-discharge (Fig. 2-1).

2. Response to methacholine in patients with pH1N1-induced acute asthmatic symptoms

Forty-nine children were subdivided according to their previous history of asthma. Nine children (18.4%) reported a previous history of asthma, and 40 children (81.6%) reported no history of asthma. No differences in blood eosinophil count, serum total IgE, or other pulmonary function parameters were observed between children with and without a previous history of asthma (Table 2-1). Among the 49 children who performed the MCT, 17 children (34.7%) were positive and 32 (65.3%) were negative. The response to methacholine 3 months following H1N1-induced acute asthmatic symptoms was negative in 29 children who did not have a previous history of asthma (Table 2-2). None of the children with a negative response to methacholine reported any recurrent wheezing or physician-diagnosed asthma during the next three years.

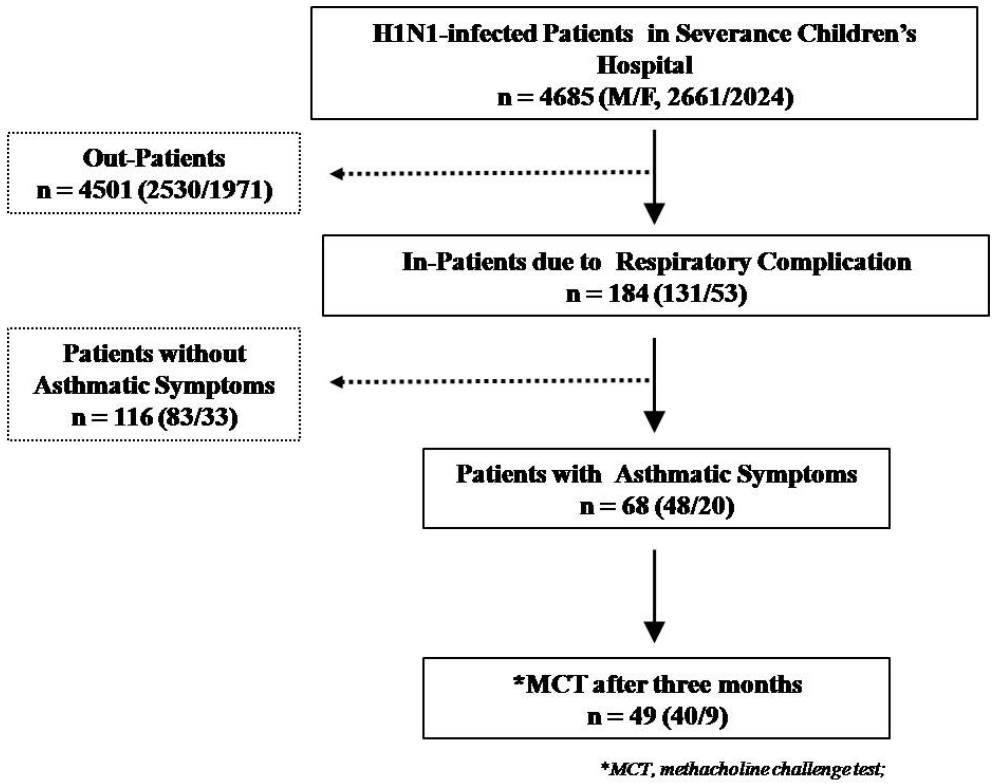


Figure 2-1. Subject demographics. Among the 4,685 patients confirmed with pH1N1 infection at Severance Children's Hospital, 184 patients (3.9%) were hospitalized due to respiratory complications, 68 of whom experienced moderate to severe acute asthmatic symptoms. Forty-nine children performed the methacholine challenge test three months post-discharge.

Table 2-1. Comparison between H1N1 induced acute asthmatic subjects with and without previous asthma history

	Previous asthma history	
	Positive	**Negative**
Sex (M/F)	6/3	34/6
Age (y)	6.7 (5 – 8.4)	8.1 (4.4 – 13.7)
BMI	17.93 ± 1.6	17.23 ± 2.6
Blood eosinophils ($log\mu L^{-1}$)	5.31 ± 1.05	5.34 ± 1.09
Serum total IgE (logkU/L)	6.46 ± 0.82	6.25 ± 1.26
FEV_1 (% pred)	95.26 ± 13.36	102.03 ± 20.62
FVC (% pred)	93.29 ± 15.67	99.72 ± 18.05
FEV_1/FVC (%)	87.73 ± 6.16	86.55 ± 7.59

BMI, body mass index; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity. Data are represented as mean ± standard deviation.

Table 2-2. Association between airway hyperresponsiveness and previous asthma history in patients with H1N1 induced acute asthmatic symptoms.

Number of Subjects (N = 49)		**Methacholine Challenge Test**		
		$PC_{20} \geq 16mg/mL$	**$PC_{20} < 16mg/mL$**	**Total**
Previous Asthma Diagnosis	Negative	29 (59.2%)	11 (22.4%)	**40 (81.6%)**
	Positive	3 (6.1%)	6 (12.3%)	**9 (18.4%)**

3. Influenza induces severe inflammation in the lung

To confirm the pathogenicity of the H1N1 influenza virus, C57BL/6 mice were infected with 1×10^4 pfu of H1N1 influenza. H1N1-infected mice exhibited a loss in body weight, and alveolar macrophages and neutrophils were accumulated in the BALF at 5 days post-infection (Fig. 2-2A, and B). Infiltrated lymphocytes were detected, but eosinophils (Fig. 2-2C) and mast cells (data not shown) were not observed. Histological features showed severe inflammation in the lung, including perivascular cuffing with infiltrated polymorphonuclear cells (Fig. 2-3A). These cells escaped into the bronchiole and accumulated in the interstitium regions. Damage to the epithelial cell walls was detected and the multi-shapes of epithelial cells were increased and expanded in H1N1-infected mice. The interstitium was thicker due to infiltration compared with the mock-infected group. Disease scores were significantly increased in the H1N1-infected group (Fig. 2-3B).

To confirm that the observed lung inflammation was induced by the H1N1 infection, inflammatory cytokine levels in the BALF were measured. Inflammatory cytokines such as IFN-γ, TNF-α, IL-6, and IL-1β as well as chemokines such as MCP-1 and RANTES were dramatically increased in the H1N1-infected mice (Fig. 2-4). These data indicate that the intranasal administration of the pH1N1 virus can efficiently infect mice and cause lung inflammation.

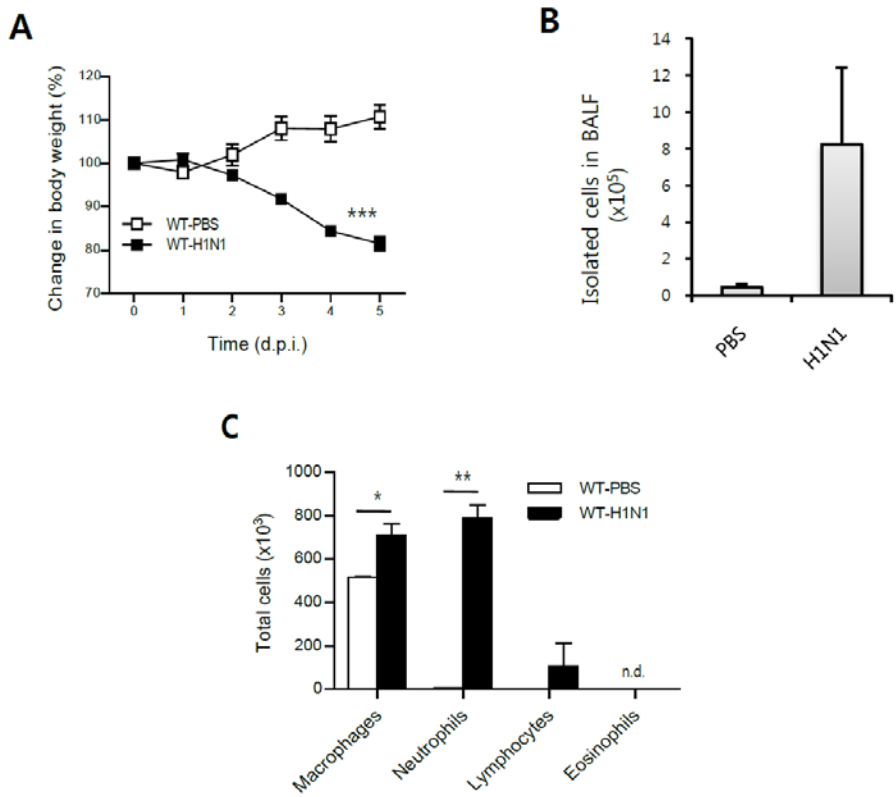
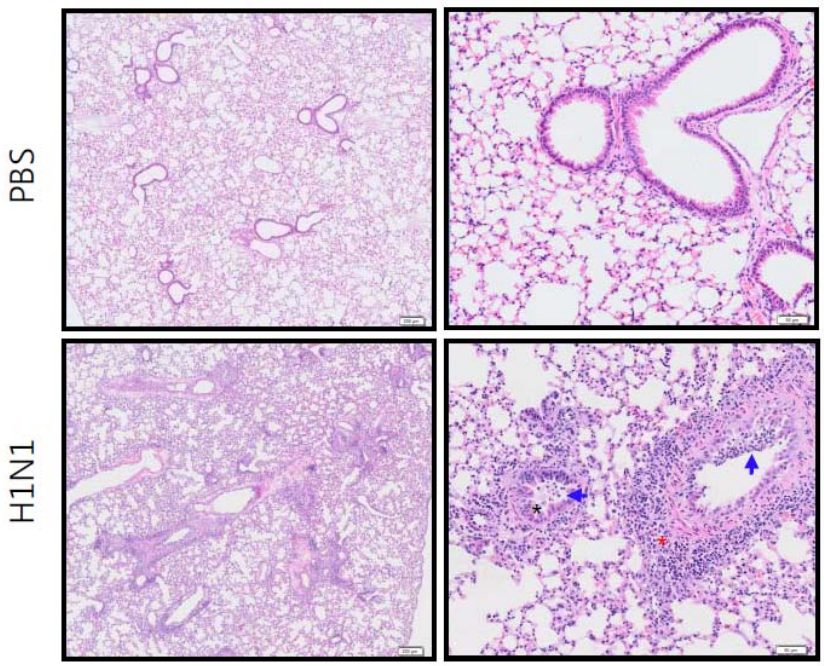


Figure 2-2. pH1N1 virus isolated from influenza-infected humans induces cell infiltration into BAL in mouse. (A) Changes in body weight in mock- and virus-infected mice. (B) Number of total infiltrated cells in the bronchoalveolar fluid. (C) The number of infiltrated inflammatory cells in the bronchoalveolar fluid following a cytospin assay. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared with the mock-infected group from the same strain. Data are represented as the mean ± SD from at least three independent experiments. n.d is not detected.

A



B

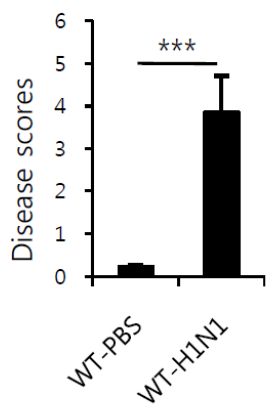


Figure 2-3. pH1N1 virus infection induces severe histological changes in lung of mouse. (A) Histological features of the lung. Lung samples taken 5 days post virus- (H1N1) or mock- (PBS) infection were stained with hematoxylin and eosin. The black asterisk indicates epithelial cell hyperplasia, the blue arrow indicates cell flooding into the cavities of the alveoli, and the red asterisk indicates perivascular cuffing with infiltrated cells. The bar indicates 200 μm (left) or 50 μm (right). (B) Disease scores based on the levels of destruction of the mucosa, epithelial cells, and alveoli, cell infiltrations into the submucosal region, epithelial cell hyperplasia, cell flooding into the interstitium, and perivascular cuffing with polymorphonuclear cells infiltration as well as other lymphoid cells using a blind test. *** $p<0.001$ when compared with the mock-infected group from the same strain. Data are represented as the mean ± SD from at least three independent experiments.

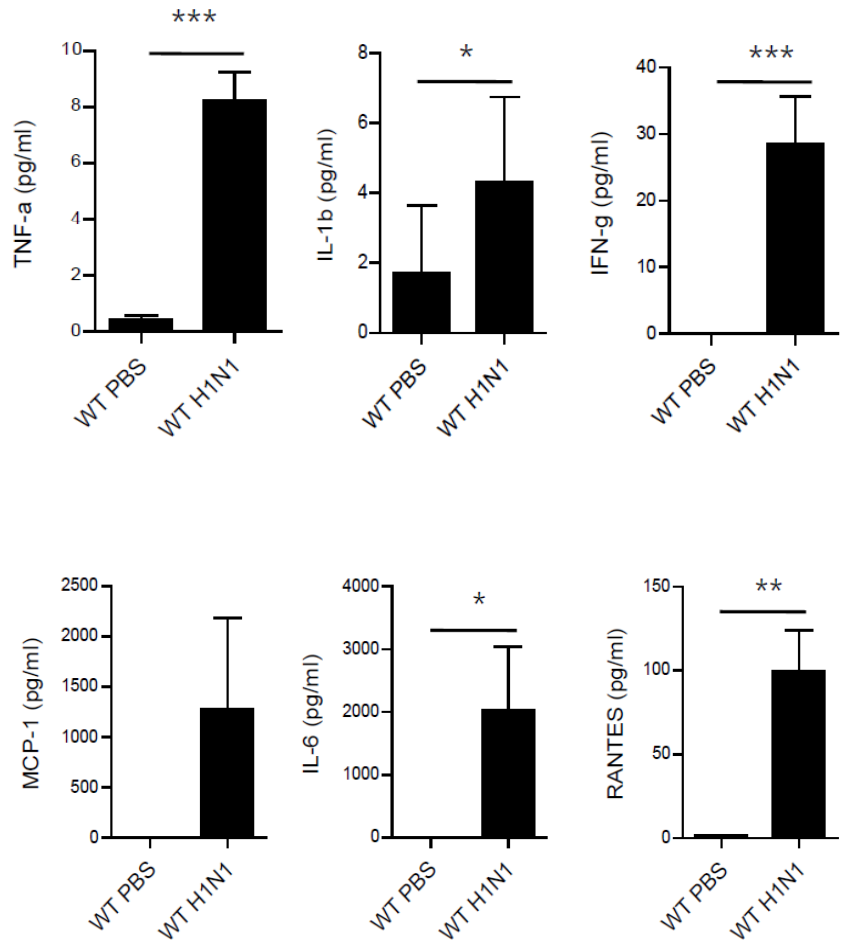


Figure 2-4 pH1N1 virus infection induces inflammatory cytokine production in mice. TNF-α, IL-1β, and IFN-γ were measured by ELISA and MCP-1, IL-6, and RANTES were measured by CBA. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared with the mock-infected group from the same strain. Data are represented as the mean ± SD from at least three independent experiments.

4. AHR in influenza-infected mice

To confirm that the pH1N1 infection directly induces asthmatic symptoms, the change in lung resistance (R_L) was measured in mice using the MCT. H1N1-infected wild-type B6 mice exhibited AHR 5 days post-infection (Fig. 2-5A). AHR is associated with Th2-type cytokines such as IL-4, IL-5, and IL-9. Increased levels of IL-4 and IL-5 were detected in the BALF in the H1N1-infected group but not the mock-infected group (Fig. 2-5B). However, the level of IL-9 was not significantly increased in either group.

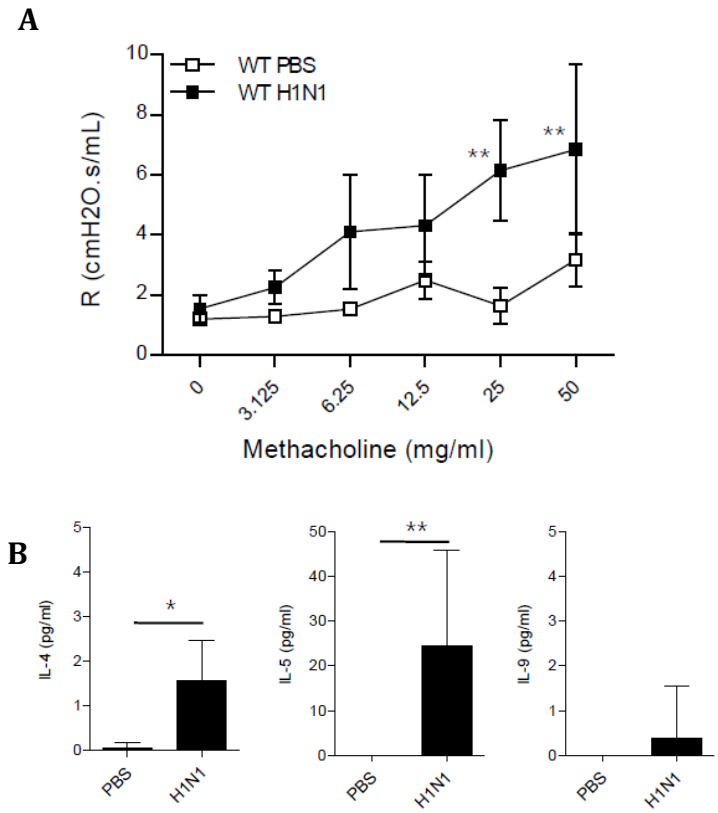


Figure 2-5. Influenza infection induces airway-hyperresponsiveness in mice. (A) Change in lung resistance (R_L) in 8-week-old C57BL/6 mice (n=5 per group) was measured by Flexivent following a challenge with serially diluted methacholine nebulized into the trachea under anesthetic conditions on day 5 post-infection with H1N1 or PBS. (B) Secreted levels of cytokines for IL-4, IL-5, and IL-9 in the bronchoalveolar fluid. Samples were separated into cells and fluid by centrifugation. Cytokines in the fluid were measured by LSRII following CBA and analyzed by the FCAP CBA analysis program. * $p<0.05$; ** $p<0.01$ when compared with the mock-infected group from the same strain. Data are represented as the mean ± SD from at least three independent experiments.

5. Influenza infection induces the activation of ILC2

An earlier study showed that the induction of AHR following H3N1 infection may involve the activation of ILC2 and cytokines produced from these cells in the lung (10). To clarify the relationships between influenza-induced AHR and the activation of ILC2, lymphocytes with the CD45 surface marker were sorted by size and analyzed for ILC2 markers. An approximately ten-fold difference in $Lin^{-}STII^{+}$ cells was observed between the two groups (0.199% in mock-infected mice and 1.31% in virus-infected mice among the CD45+ cells), and the level of $sca\text{-}1^{+}c\text{-}kit^{+}$ cells was increased from 1.45% in the mock-infected group to 17.7% in the virus-infected group. Moreover, $sca\text{-}1^{+}CD25^{+}$ cells also were increased from 0.513% in the mock-infected group to 2.38% in the virus-infected group (Fig. 2-6). To further confirm the activation of ILC2, the mRNA expression levels of IL-4, IL-5, and IL-13 were analyzed. IL-4, IL-5, and IL-13 expression levels were significantly increased in Lin^{-} cells but not in total lung (Fig. 2-7). These results indicate that activated Lin^{-} cells produce type 2 cytokines, which supports the role of ILC2 in inflammatory responses following pH1N1 infection. In addition, IL-33 is known to prime Th2-type cytokine induction and AHR. The level of IL-33 was measured in the whole lung. Significantly increased levels of IL-33 were observed in the virus-infected group (Fig. 2-8). Taken together, these results suggest that influenza induces activation of ILC2, increased Th2 cytokine mRNA levels, and IL-33 production levels in the lung.

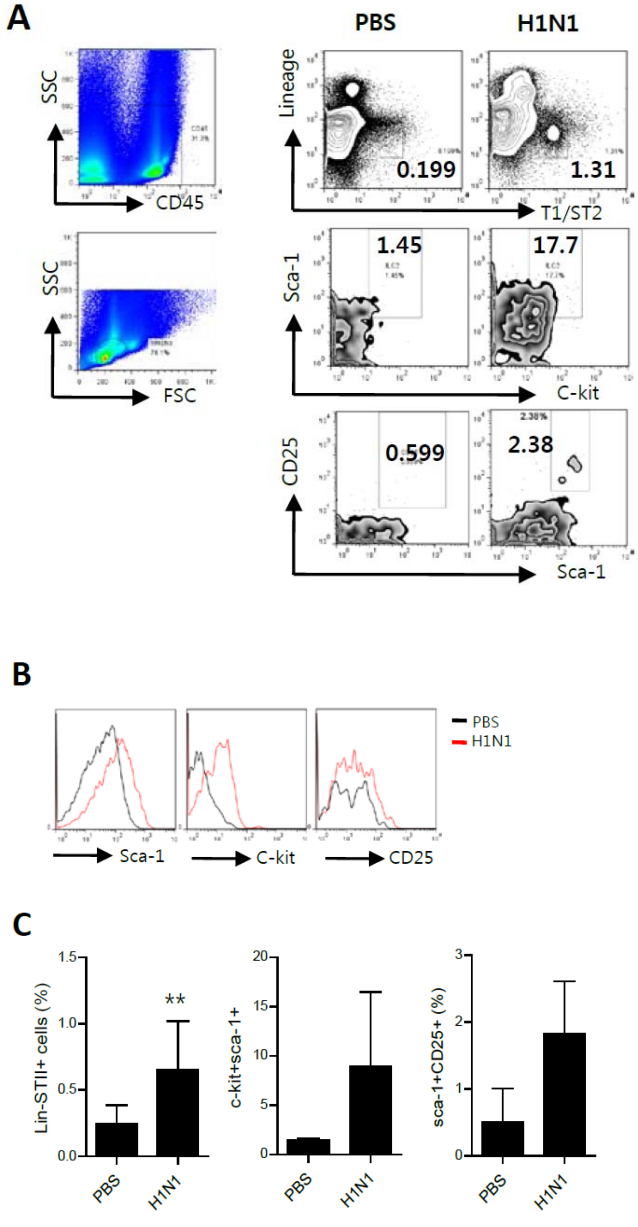
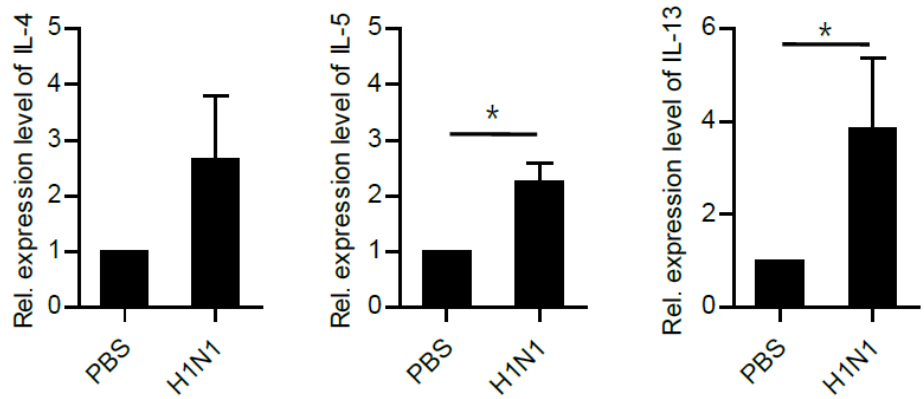

A
PBS
H1N1
SSC
CD45
SSC
FSC
Lineage
T1/ST2
0.199
1.31
Sca-1
C-kit
1.45
17.7
CD25
Sca-1
0.599
2.38
B
PBS
H1N1
Sca-1
C-kit
CD25
C
Lin-STII+ cells (%)
c-kit+sca-1+
sca-1+CD25+ (%)
**
PBS
H1N1

Figure 2-6. pH1N1 virus infection results in an increase in ILC2 in the lung. (A) ILC2 in the lung. The gating strategy for ILC2 isolated from total $CD45^+$ lung lymphoid cells. The numbers in the box indicate the percentage of cells in each gate. SSC, side scatter; FSC, forward scatter. The top panels show increased levels of $CD45^+Lin^-ST2^+$ cells in the lung. The lower panels indicate the expressed levels of sca-1, c-kit, and CD25 from gating on the Lin^-ST2^+ subset in the top panels. The numbers indicate the percentage of cells in the quadrangle in each panel. (B) Panel A) expressed as a histogram. (C) The percentage of Lin^-ST2^+ cells, $Lin^-ST2^+c\text{-}kit^+sca\text{-}1^+$ cells, and $Lin\text{-}ST2^+sca\text{-}1^+CD25^+$ cells. Each population was statistically analyzed using data from at least three independent experiments. ** $p<0.01$; when compared with the mock-infected group from the same strain.

A.



B.

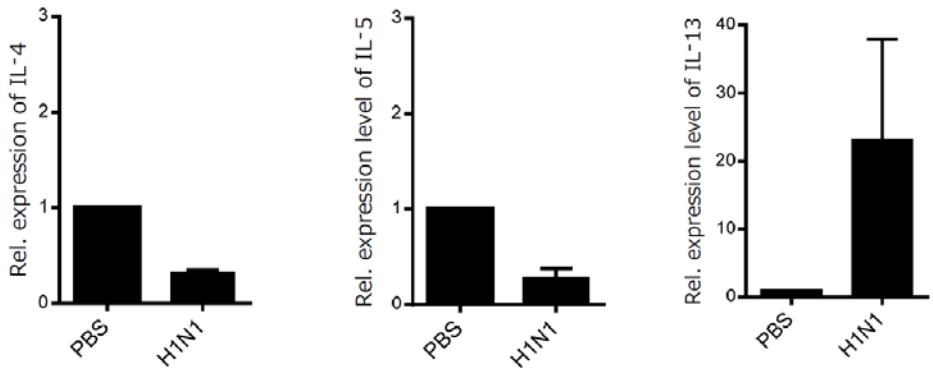


Figure 2-7. The mRNA expression levels of IL-4, IL-5, and IL-13. **(A)** Lin$^-$ cells were isolated by negative sorting using MACS after Lineage staining. **(B)** mRNA were isolated from total lung. The levels of mRNA expression are displayed as the expression levels of each target gene relative to the ct value of expressed GAPDH. * $p<0.05$ when compared with the mock-infected group from the same strain.

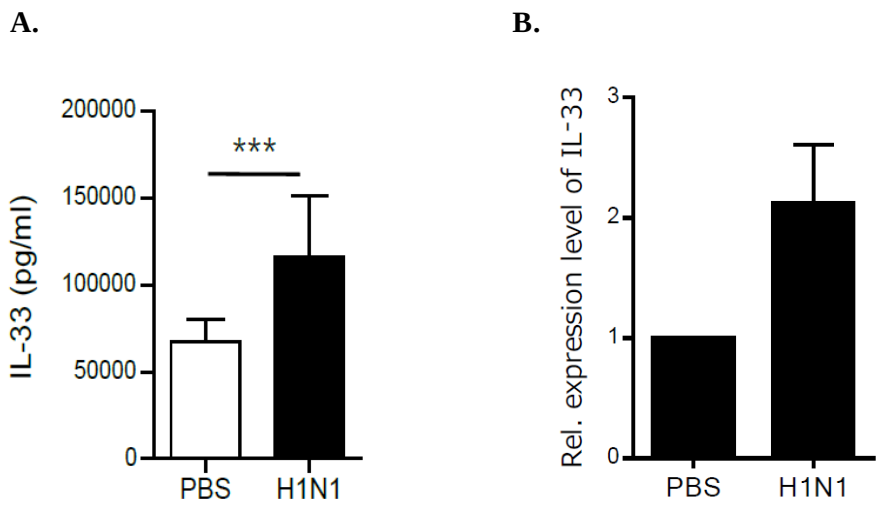


Figure 2-8. The levels of IL-33 in lung of mice. **(A)** Lungs were homogenized and supernatant were used for detection of IL-33 using ELISA. **(B)** mRNA level of IL-33 in total lung. The results are expressed as the mean ± SD from three different experiments. *** $p<0.001$ when compared with the mock-infected group from the same strain.

6. Influenza infection induces AHR independent from adaptive immunity

To exclude the possibility of an involvement of the adaptive immune response, including the Th2-cell secreting type 2 cytokines, $Rag1^{-/-}$ mice that lack conventional T and B cells were infected with the pH1N1 virus or PBS. Infected $Rag1^{-/-}$ exhibited similar levels of inflammation in response to pH1N1 infection as wild-type mice (Fig. 2-9- 2-11). Histological features showed severe inflammation in the lung, including perivascular cuffing with infiltrated PMN cells as shown in the wild-type mice (Fig. 2-10). Increased levels of inflammatory cytokines and type 2 cytokines such as IL-4, and IL-5 in the BALF and IL-33 in the lung also were detected in $Rag1^{-/-}$ mice (Fig. 2-12A and B). AHR was observed in the pH1N1-infected group only (Fig. 2-13). These results therefore indicate that $Rag1^{-/-}$ mice experience all the same symptoms associated with pH1N1 infection as wild-type mice. These results suggest that pH1N1-induced acute AHR is not related to an adaptive immune response such as Th2 immunity.

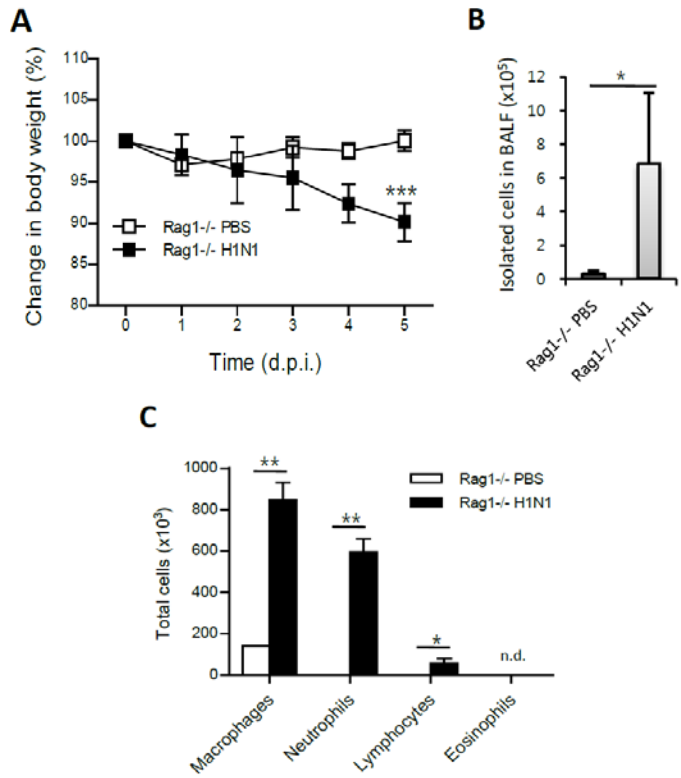
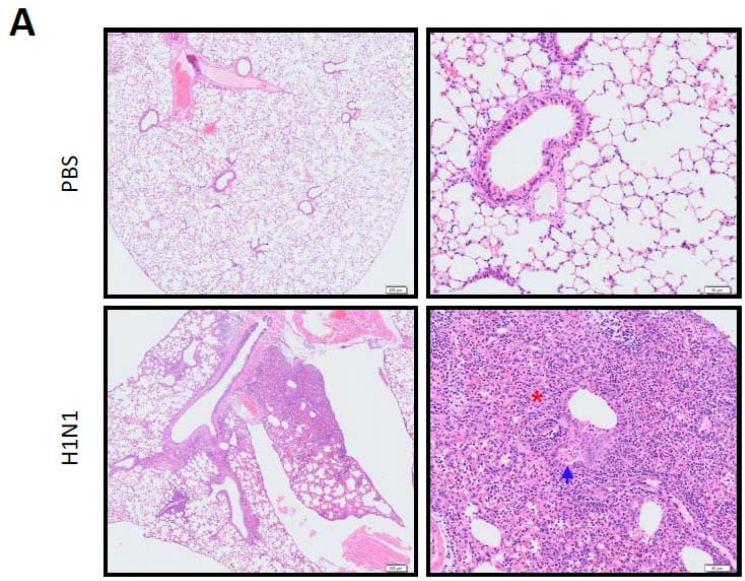


Figure 2-9. pH1N1 virus infection induces cell infiltration in Rag1$^{-/-}$ mice. **(A)** Changes in body weight in mock- and virus-infected Rag1$^{-/-}$ mice. **(B)** Number of total infiltrated cells in the bronchoalveolar fluid. **(C)** The number of infiltrated inflammatory cells in the bronchoalveolar fluid following a cytospin assay. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared with the mock-infected group from the same strain. Data are represented as the mean ± SD from at least three independent experiments. n.d is not detected.


A
PBS
H1N1

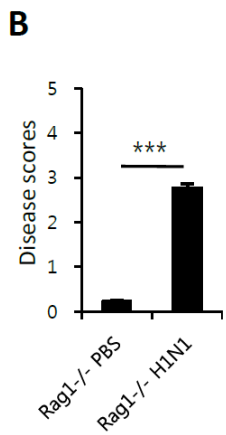

B
Disease scores
5
4
3
2
1
0
***
Rag1-/- PBS
Rag1-/- H1N1

Figure 2-10. pH1N1 virus infection induces severe lung inflammation in $Rag1^{-/-}$ mice independent from the adaptive immune response. **(A)** Histological changes in the lung of $Rag1^{-/-}$ mice. The blue arrow indicates epithelial cell hyperplasia, the red asterisk indicates cell flooding into the cavities of the alveoli, and the red asterisk indicates perivascular cuffing with infiltrated cells. The bar indicates 200 μm (left) or 50 μm (right). **(B)** Disease scores based on the levels of destruction of the mucosa, epithelial cells, and alveoli, cell infiltrations into the submucosal region, epithelial cell hyperplasia, cell flooding into the interstitium, and perivascular cuffing with polymorphonuclear cells infiltration as well as other lymphoid cells using a blind test. *** $p<0.001$ when compared with the mock-infected group from the same strain. Data are represented as the mean ± SD from at least three independent experiments.

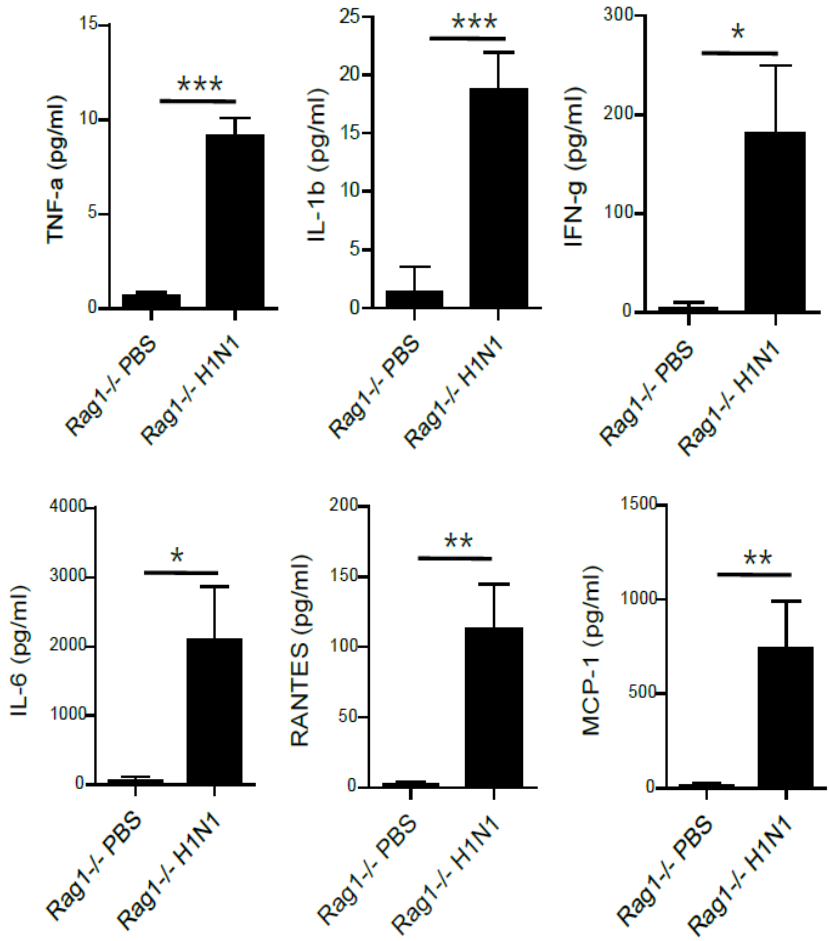


Figure 2-11. pH1N1 virus infection induces inflammatory cytokine production in Rag1$^{-/-}$ mice. TNF-α, IL-1β, and IFN-γ were measured by ELISA and IL-6, RANTES, and MCP-1 were measured by CBA.* $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared with the mock-infected group from the same strain. Data are represented as the mean ± SD from at least three independent experiments.

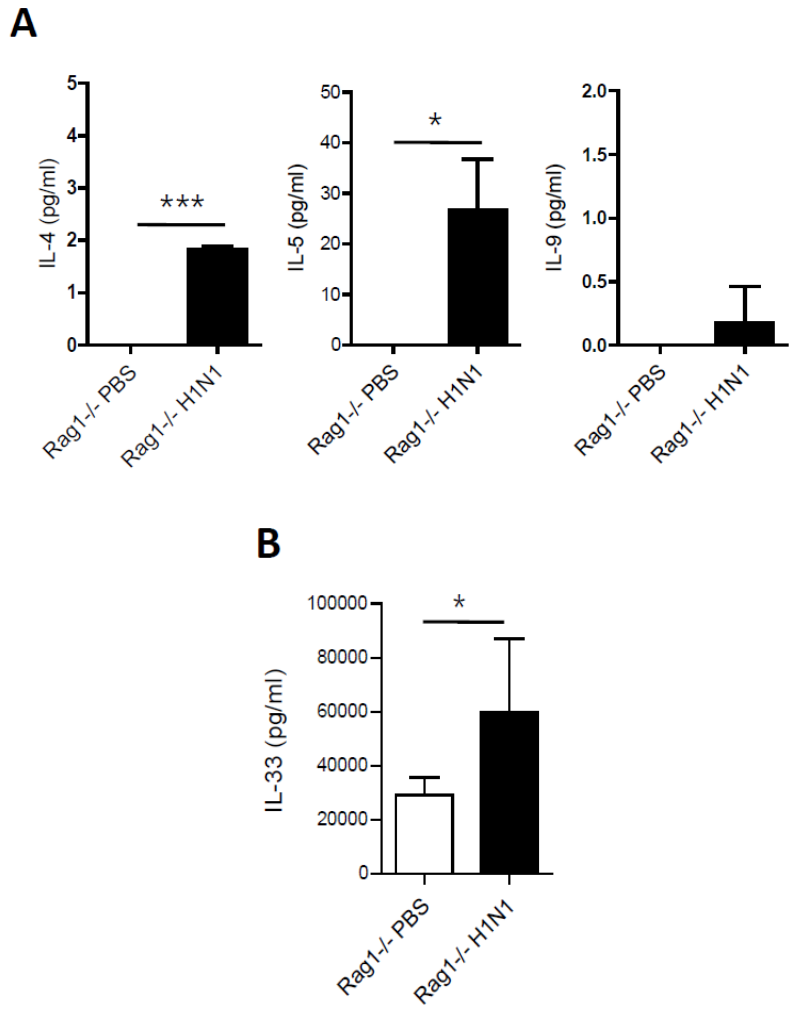


Figure 2-12. pH1N1 virus infection induces Th2 type cytokines production and IL-33 in the lung of Rag1$^{-/-}$ mice. (A) Cytokine levels of IL-4, IL-5, and IL-9 in the bronchoalveolar fluid. (B) Cytokine levels of IL-33 in the lung. * $p<0.05$; *** $p<0.001$ when compared with the mock-infected group from the same strain. Data are represented as the mean ± SD from at least three independent experiments.

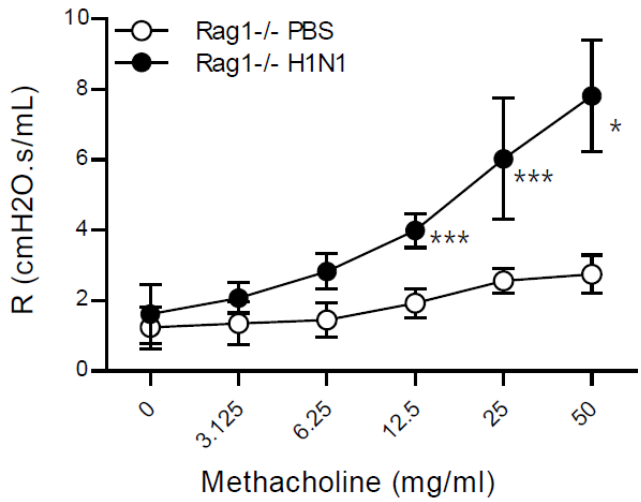


Figure 2-13. Change in lung resistance (R_L) in $Rag1^{-/-}$ mice following H1N1 infection. R_L was measured by Flexivent following a challenge with serially diluted methacholine nebulized into the trachea under anesthetic conditions on day 5 post-infection with H1N1 or PBS. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared with the mock-infected group from the same strain. Data are represented as the mean ± SD from at least three independent experiments.

V. Discussion

pH1N1 was recorded as the first influenza pandemic of the 21st century. This single institution study revealed the high prevalence of H1N1 infection in young people during the worldwide pandemic period (20,21). In this study, 184 children were hospitalized due to respiratory complications, 64 of which had acute asthmatic symptoms. Forty-nine of these patients were tested three months post-discharge, and more than 70% of the children who did not have a previous history of asthma were negative on the MCT. Laitinen *et al*. previously reported that influenza infection causes transient bronchial hyperresponsiveness in healthy adults (22).

Hasegawa *et al*. has documented that the pandemic influenza virus infection greatly increased the risk of asthma exacerbation, pneumonia, and atelectasis compared with the seasonal influenza virus (9). Dahl and colleagues reported that infection with the influenza A virus affects the subsequent development of allergen-induced lung inflammation in a mouse model (3). Chang *et al*. reported the appearance of AHR following H3N1 virus infection (10), and suggested that the main cause of AHR following H3N1 infection is an innate immune cell that does not originate from the common lymphoid precursor in the lung. The present study confirmed that pH1N1 successfully induces lung inflammation in both of wild-type and $Rag1^{-/-}$ mice. This infection also induces several inflammatory cytokines, including MCP-1, IFN-γ, RANTES, IL-6, TNF-α, and IL-1β (Fig. 2-4 and 2-11) (23,24). The present study also confirmed that pH1N1 infection induced

AHR in mice independent from an adaptive immune response (Fig. 2-5 and 2-13).

Influenza-induced AHR differs from asthma, which requires repeated allergen sensitization and activates Th2 cells leading to eosinophil influx and mast cell activation (25). The induction of AHR by the pH1N1 infection was the result of an acute response against the virus in the lungs of mice. No eosinophils (Fig. 2c) or other cells such as basophils or mast cells were found (data not shown). Additionally, the detection of increased levels of IL-5 and IL-4 but not IL-9 with inflammatory cytokines secretion supports the hypothesis of ILC2 involvement in influenza-induced AHR (Fig. 2-5, 2-7 and 2-12). As expected, ILC2 activation markers such as sca-1 and c-kit and CD25 expression levels on these cells as well as the total number of $Lin^{-}ST2^{+}$ cells all were increased. These results differ from a previous report in which only the level of ILC2 activation without a change in total population was observed (10). The levels of $c\text{-}kit^{+}sca\text{-}1^{+}$ cells were increased in the pH1N1-infected group in a range of approximately 2 to 20% because the expression level of c-kit varied significantly across each experiment. Therefore it was speculated that the c-kit molecule was not a sufficient activation marker for ILC2. To further confirm increased ILC2 as a potent effector of Th2-type cytokine secretion, the relative expression levels of IL-4, IL-5, and IL-13 mRNAs were detected by real-time PCR. As a second group of ILCs, the characteristics of these cells resemble Th2 cells, which mediate allergic responses that secrete type 2 cytokines and express transcription factor GATA3 (14). As shown in Figure 2-7, increased levels of IL-4, IL-5, and IL-13 mRNA expression were observed in Lin^{-} cells following infection with the influenza virus. It is

assumed that the type 2 cytokines expressed in Lin^- cells following infection play a role as potent effectors of influenza-induced AHR in this study.

According to several studies, IL-33 has an important role as one of the earliest released signaling molecules following epithelial damage to orchestrate the recruitment and activation of responder cells (26,27). Haenuki *et al.* reported that experimental allergic rhinitis is not induced in $IL\text{-}33^{-/-}$ mice following a ragweed pollen challenge compared with control wild-type mice (28). Moreover, Préfontaine *et al.* has observed an increased level of IL-33 gene and protein expression in endobronchial biopsies from subjects with asthma (29). Furthermore, anti-IL-33 antibody treatment inhibits airway inflammation in mice (30). In this study, increased levels of IL-33 were detected in the lung homogenate of influenza-infected mice (Fig. 2-8 and 2-12B). Increased IL-33 therefore may directly or indirectly influence AHR and influenza-induced inflammation in mice.

In summary, the present study demonstrated that the pandemic H1N1 influenza infection directly induces asthmatic symptoms in patients regardless of their prior history of asthma. Experiments in mice revealed that pH1N1 infection stimulates the rapid development of AHR and Th2-type cytokine secretion in mice via the activation of ILC2. These responses may be activated independently from adaptive immunity.

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Chapter III.

H1-specific Indirect ELISA for Serological Detection of Canine Influenza Virus H3N2 Infection in Dogs

I. Abstract

Indirect ELISA system using a recombinant HA1 protein of Canine influenza virus (CIV) H3N2 as coating antigen was developed and characterized to apply for the potential use of serosurveillance against virus in dog. CIV H3N2-specific indirect ELISA was established using recombinant HA1 protein (baculovirus-expression system) as a coating antigen. A total 65 CIV H3N2-positive or negative canine sera were tested by the indirect ELISA for ROC analysis and compared with HI test. The archived canine sera were collected 10 days after intranasal inoculation of canine H3N2, seasonal H3N2 (A/Brisbane/10/2007) and pandemic H1N1 influenza virus (A/California/04/2009), respectively, were used for the cross-reaction test. Adjusted O.D of 0.36 was determined to be a optimal cut-off value for seropositivity. Also, indirect ELISA showed 93.5% and 94.7% of sensitivity and specificity, respectively when compared to HI test. Cross-reaction test was also performed using the archive canine sera related with CIV H3N2, seasonal H3N2 (human) and pandemic H1N1 (human) influenza viruses. Based on the data, the HA1 of CIV H3N2-specific indirect ELISA can be used to survey the canine H3N2-specific IgG in large scale with simple procedure whose result is comparable with HI test.

II. Introduction

Since interspecies transmission of avian influenza virus (H3N2) to dogs was found, the intraspecies transmission of canine influenza virus (CIV H3N2) was also proved by experimental and surveillance studies (1-3). The previous studies for the virus (CIA H3N2) serology were based on the heamagglutination inhibition (HI) test or commercialized competitive ELISA system which was established to detect universal avian influenza A virus-specific antibodies using NP protein (3). However, there were some problems for using those methods for CIV H3N2 serology. While the HI test is labor-intensive, the commercialized competitive ELISA, with its own systemic characterization, cannot clarify that the positive result due to canine H3N2 or other avian influenza virus. However, the previous study showed that hemagglutinin (HA)-specific ELISA system was successful to detect avian influenza A (H5N2) virus-specific antibodies, which was closely related to the result of subtype-specific HI and microneutralizing assay (4). Therefore, in this study, indirect ELISA system using a recombinant protein of CIA H3N2 as coating antigen was developed and characterized to apply for the potential use of serosurveillance against the virus in dog.

III. Materials and methods

1. Antigen preparation for the indirect ELISA

The 5'-oligonucleotide (5'-CAGGATCCCAATCTTCCAGGAAATGAAAATAA) contained a BamHI site and the coding sequence for the N-terminus amino acid residues of canine H3N2 HA1. The 3'-oligonucleotide (5'-CAGCGGCCGCTCAATGGTGATGGTGATGATGGGTTTGCCTCTCAGGGAC) contained a NotI site, stop codon, C-termianl 6XHis tag, and the encoding sequence for the C-terminus amino acid residues of Canine H3N2 HA1 (A/canine/Korea/GCVP01/2007(H3N2)). Canine H3N2 HA1 coding sequence was amplified by PCR using two oligonucleotides as described above. The PCR product was purified with a QIAquick PCR purification kit (Qiagen, Boston, MA), restricted with BamHI and NotI, repurified with a QIAquick PCR purification kit (Qiagen), and subcloned in BamHI-NotI restricted pAcGP67A, which resulted in pAcGP67A-cH3N2 HA1.

The pAcGP67A-cH3N2 HA1 DNA (2 μg) was co-transfected with 0.5 μg of BaculoGold AcNPV DNA (BD Bioscience) into 2 x 10^6 sf9 cells following the recommendations of the supplier. On day 5 of post-transfection, the medium was centrifuged and the supernatant was tested in a limiting dilution assay with sf9 cells. A total of 2 x 10^6 sf9 cells were incubated with 10-fold dilutions of supernatant in 6-well plates. Eight days post-infection, medium was centrifuged and the supernatant was tested in a limiting dilution assay with sf9 cells. 2 x 10^6 sf9 cells were incubated with 10-fold dilutions of supernatant in T-75 Flask. Recombinant virus in the

supernatant was then amplified by infecting sf9 cells. A total of 2 x 10^6 High5 cells in Sf-900 II SFM medium were infected with recombinant baculovirus in spinner flasks at m.o.i. of 0.01-10. Supernatant was harvested after three days post infection. The supernatant was centrifuged at 3000 g for 10 min to remove cell debris. The supernatant was loaded on Ni-NTA resin equilibrated with 20 mM Tris-HCl (pH8.0), 0.5M NaCl, and 5mM imidazole. It was washed the resin with 5mM imidazole and eluted with 250mM Imidazole. The eluted protein, canine H3N2 HA1, was dialysed with 20 mM Tris-HCl (pH8.0) and 10% glycerol.

2. Indirect ELISA

Indirect ELISA system was optimized with the principle that HA-specific canine IgG was detected by sheep anti-dog and anti-sheep IgG-HRP. ELISA plates (Greiner bio-one, Frickenhausen, Germany) were coated with canine H3N2-derived HA1 (50 ng/well) in cold carbonate buffer (pH 9.6) and incubated overnight at 4 ◦C. Antigen-free carbonate buffer were coated for endpoint assay. 0.2% skim milk in phosphate buffered saline containing 0.05% tween 20 (PBS-T) was used for blocking (37 °C for 1 h). After washing the plates, 100-fold diluted canine serum samples with blocking buffer containing 10 ㎕/ml of hi 5 cell culture supernatant then were applied to the duplicate to each antigen coated and non-coated wells and incubated for 90 min at 37 ◦C. HRP-conjugated sheep anti-dog IgG Ab was added to each well and incubated for the same condition as the above step. For color development, 100 ㎕ of TMB microwell peroxidase substrate contains 3,3’,5,5’-tetra-methylbezidine in acidic buffer (KPL; Gaithersburg, MD) was

added to each well and incubated for 5 min at RT. To stop reaction, equal volume of manufacture's recommended solution (2.5 N H_2SO_4) was added, and the plates were read at a wavelength of 450 nm within 30 min. Endpoint titers of canine H3N2-HA-specific IgG Abs were expressed as the OD value of HA uncoated well subtraction from HA coated well (adjusted OD).

IV. Results

1. The established indirect ELISA was superior then competitive ELISA and sensitive than HI test

Experiment 1. The established indirect ELISA was compared with HI test. A total of 65 canine sera were tested by the indirect ELISA. The sera were provided by Animal and Plant Quarantine Agency, Anyang, Korea and Green Cross Veterinary Products Company, Yong-In, Korea. While the negative sera (44 experimental dog's sera) were determined when HI titer was less than 10, the positive sera were consisted of 16 experimental dog's sera (160~>1280 HI titers) and 5 anti-sera (160~640 HI titers) against canine influenza H3N2 virus. The HI test was performed with 8 HA unit of canine influenza virus (H3N2) (A/canine/Korea/GCVP01)/2007) following the OIE (Office International des Epizooties; became the World Organization for Animal Health) manual.

The ROC analysis was performed with web-based Receiver Operating Characteristic (ROC) analysis tool (5).

Experiment 2. To validate the obtained cut-off value, an antiserum against canine H3N2 which was kindly provided by Green Cross Veterinary Products Company was serially diluted to make sample with 40, 80, 160, and 320 HI titers. Since these HI titers were in the fine line between seropositive and seronegative decision with HI test, the prepared sera were tested as

duplicate with indirect ELISA.

Experiment 3. The archived canine sera (10 sera) which were collected 10 days after interanasal inoculation of canine H3N2, seasonal H3N2 (A/Brisbane/10/2007) and pandemic H1N1 influenza virus (A/California/04/2009), respectively, were used for the cross-reaction test. The individual serum was tested with HI test, commercialized competitive ELISA and indirect ELISA system. The competitive ELISA (Bionote LtD., Hwaseong, Korea) was performed following the manual and its result was expressed with positive or negative (positive if the PI value was above 50 and negative if the value was less than 50). The canine sera were obtained from the previous animal experiment, which was approved by Institutional Animal Care and Use Committee (IACUC) of Korea Research Institute of Bioscience and Biotechnology (KRIBB; KRIBB-AEC-12054).

Regarding HI test as a gold standard, ROC curve was drawn in figure 3-1. The area under curve (AUC) value was 0.993. When the cut-off value was determined to 0.17, sensitivity and specificity were 95.7% and 94.7%, respectively. When the cut-off value was determined to 0.36, sensitivity and specificity were 93.5% and 94.7%, respectively. Considering microscopic discrimination between negative and positive reactions, for indirect ELISA in this study, the cut-off value was determined 0.36 of adjusted O.D.

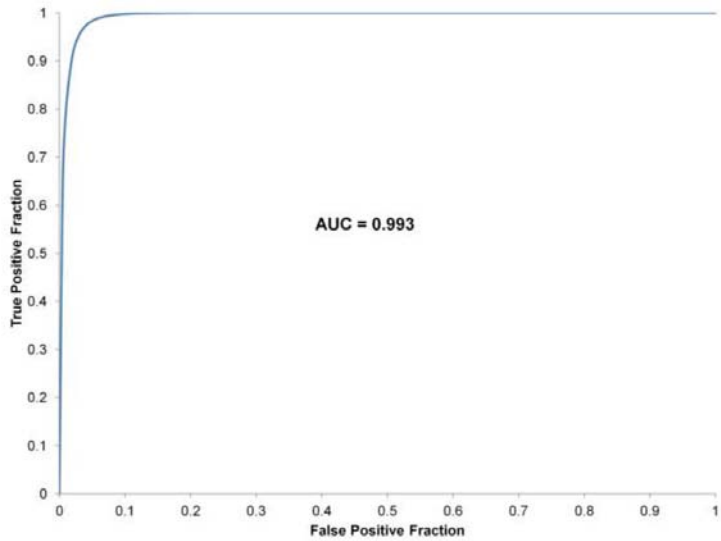

Figure 3-1. Receiver Operating Characteristic (ROC) curve analysis of the indirect ELISA with HI test.

In addition, the indirect ELISA was compared with HI test using sera with low range of HI titers. When the diluted canine sera with 40, 80, 160, and 320 HI titer were tested with indiect ELISA, the adjusted O.D more than 0.36 began to be found from the diluted serum with 320 HI titers (Figure 3-2). Based on the data, the cut-off value of the ELISA system was closely related with 160~320 range of HI titers.

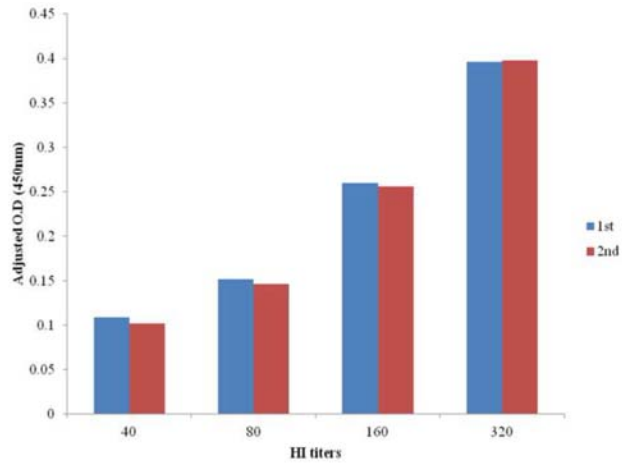

Figure 3-2. Comparative analysis between HI test and indirect ELISA in the diluted serum with low range of HI titers. * Cut-off value was determined as 0.35.

Finally, seropositives among HI test, competitive ELISA and indirect ELISA were compared using the canine sera related with different influenza viruses. Two of three canine sera collected 10 days post canine H3N2 innoculation were positive with HI test showing 320 and 640 of HI titers, respectively, while all sera were showen to be positive with competitive ELISA and indirect ELISA system (Table 3-1). In the case of canine sera from seasonal H3N2 and pandemic H1N1-innoculated dogs, HI titers of all the sera were below 10. The competitive ELISA showed that 3/3 and 2/4 of sera from seasonal H3N2 and pandemic H1N1-inoculated dogs were positive, respectively, while indirect ELISA did 1/3 and none were positive in the same cases.

Table 3-1. Competitive seropositivities of canine sera collected 10 days after different influenza virus inoculation using HI test, competitive commericial ELISA and indirect ELISA in this study.

Serum information		HI test *	Competitive	Indirect
Innoculated virus	**Individuals**		**ELISA**	**ELISA**
	U-45	320	+	+ (0.75)
Canine H3N2	U-46	<10	+	+ (0.36)
	U-54	640	+	+ (0.74)
	U47	<10	+	+ (0.72)
Seasonal H3N2	U-52	<10	+	- (0.00)
	U-51	<10	+	- (0.00)
	U48	<10	+	- (0.04)
Pandemic H1N1	U-40	<10	+	- (0.03)
	U-59	<10	-	- (0.01)
	U-53	<10	-	- (0.02)

* Canine H3N2 as an antigen

V. Discussion

The CIV H3N2-specific antibodies can be detected by HI test, since there has been no CIA H3N2-specific ELISA system. A commercialized competitive ELISA kit (universal use against avian influenza A viruses) has been used for CIV H3N2 serology in the previous studies (6, 7), but it could not clarify if the positive reaction was due to CIV H3N2. Therefore it could be assumed that it might be due to CIV H3N2 because no canine influenza viruses are evident except for CIV H3N2 in Korea. However, recently found canine influenza virus (H3N1) which was thought be a reassortment between CIV H3N2 and pandemic H1N1 influenza virus reinforces the need of subtype-specific ELISA system in dog as well (8). The HA1 protein of influenza virus was reported to be subtype-specific (9), therefore, in this study, recombinant HA1 protein was used as a coating antigen for ELISA which targeted to detect canine H3N2 influenza specific IgG.

The HA1 domain of HA was also known to be related with virus-neutralizing activity (10, 11) and several ELISAs have used the HA1 protein as the coating antigen (9, 12). In this study, indirect ELISA system was successfully established and tested with canine sera in Korea. It was highly correlated with HI titer showing 0.993 of AUC value in the ROC analysis. The cut-off value was determined as 0.36 of adjusted O.D value showing 93.5% and 94.7% of sensitivity and specificity, respectively. Since the minimum HI titer of positive sera in the ROC analysis was 160, additional ELISA test was performed using diluted canine sera of 40, 80, 160 and 320 HI titers.

Interestingly, the cut-off value was closely related with the sera between 160 and 320 HI titers. These data showed that the established indirect ELISA system is highly correlated with HI test, which may indicate the ELISA in this study is highly strain-specific.

In addition, the archived canine sera which were collected 10 days after inoculation of CIV H3N2 as well as seasonal H3N2 and pandemic H1N1, respectively, were tested with the indirect ELISA, commercialized competitive ELISA and HI test. All tests were positive for the sera from CIV-H3N2-innoculated dogs. While HI test showed negative all for the sera from seasonal H3N2-innoculated dogs, commercialized ELISA showed positive all, indicating that the commercial ELISA was cross-reactive. The indirect ELISA seemed to be somewhat cross-reactive for seasonal H3N2 because one of three sera was positive by the indirect ELISA. However, the indirect ELISA was not cross-reactive for pandemic H1N1 as the HI test was, while the commercial ELISA still showed positive reaction for the sera from pandemic H1N1-innoculated dogs. These may imply that indirect ELISA in this study was sufficiently subtype-specific, which may be similar to HI test.

In conclusion, this study demonstrated that HA1 protein of CIV H3N2 can be applied to indirect ELISA system. The indirect ELISA showed high sensitivity and specificity so that it can be used to survey the canine H3N2-specific IgG in large scale with simple procedure. Moreover, as IgGs detected by the indirect ELISA in this study would be correlated with the neutralizing antibodies due to antigen property (HA1), thus it can be alternative test of classical HI test.

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인플루엔자(H1N1) 감염에 의해 유발되는 천식증상과 개 인플루엔자, cH3N2, 특이항체 진단을 위한 간접효소면역측정법에 대한 연구

심두희

(지도교수: 유상렬)

인플루엔자바이러스의 감염은 사람에게 급성기도염증반응을 일으키는 한 원인이 된다. 새로 출몰한 바이러스 종 influenza A/California/04/2009(pH1N1)은 고병원성으로 21세기 최초로 전 세계적인 인플루엔자 대유행을 일으켰다. 이 바이러스에 감염된 환자들에게서 천식 증상과 같은 다양한 합병증이 나타나는데, 어떻게 그런 증상이 일어나는지에 대해서 아직 밝혀진 바가 없다. 몇몇 연구논문에서는 동물실험을 통해 마우스에서는 인플루엔자바이러스의 감염이 천식증상의 원인이 될 수 있다고 언급하고 있으나, 기존에 천식이나 알레르기 환자가 아닌 일반인들에게서도 pH1N1이 감염되었을 때 기도과민성반응이 나타나는지에 대한 논문은 아직까지 없다.

본 논문의 첫 번째 연구에서는 pH1N1이 감염된 어린이들(15세 이전)에게서 천식증상이 나타나는 정도와 동물모델을 이용하여 pH1N1의 감염과 기도과민성반응의 상관관계에 대해 조사·관찰해 보았다. 2009년 10월부터 2010년 2월 사이에 pH1N1에 감염되어 세브란스 병원에 입원한 어린이 4,685명을 대상으로 하여 천식증상이 있는지 여부를 조사한 결과 184명에게서 천식증상을 보이고 있음을

확인하였다. 이후, 기존의 천식증상이 있는 어린이와 그렇지 않은 어린이를 나누어, 인플루엔자가 완쾌된 3개월 후의 기도과민반응성을 알아볼 수 있는 메타콜린시도시험(Methacholine challenge test, MCT)을 실시하였다. 그 결과, pH1N1에 감염될 당시 알레르기성 기관지염 증상을 보이던 어린이의 70%가 기존의 알레르기나 천식증상이 없었던 이들로 인플루엔자가 완쾌된 후에는 폐기능이 정상으로 돌아오는 것을 확인하였다. 이들을 상대로 이후 3년간 더 추적조사를 실시하였으며, 이들 중 그 누구도 천식이나 알레르기 증상을 나타내지 않는다 것을 재차 확인하였다. 위와 같이 임상시험을 통해 천식 환자가 아닌 일반인에게서도 pH1N1 감염 후 알레르기 반응의 지표 중 하나인 기도과민성반응이 나타날 수 있음을 알 수 있었다. 다음으로, 정상마우스와 획득면역반응이 결핍된 마우스($Rag1^{-/-}$)를 사용하여 인플루엔자 바이러스를 감염시킨 후 기도과민성반응이 일어나는지 여부와 이 반응에서 자연면역반응의 역할은 무엇인지 알아보았다. 그 결과 인플루엔자바이러스의 감염이 직접적으로 급성기도과민성반응을 유도한다는 것을 알 수 있었고, 이와 동시에 폐조직에서 사이토카인 IL-33이 증가하고 선천성림프구2(ILC2)의 수와 활성도가 높아지는 것을 확인하였다. 또한 T림프구와 B림프구가 결핍된, 즉 자연면역반응만 존재하는 $Rag1^{-/-}$마우스에서도 정상마우스와 같은 결과를 얻을 수 있었으며, 이는 급성기도과민성반응이 획득면역이 없는 상황에서도 발생할 수 있음을 나타낸다. 위와 같이 동물실험을 통해 pH1N1이 일으키는 기도과민성반응과 Th2사이토카인의 증가는 ILC2의 활성화를 통해서 이루어진다는 결과를 도출해 낼 수 있었다.

두 번째 연구에서는 개들에게 감염되는 인플루엔자바이러스 H3N2 (cH3N2)의 특이적인 항체를 검출할 수 있는 간접효소면역측정법을

개발한 것에 대한 서술이다. 이러한 간접효소면역측정법은 개의 혈청에 존재하는 항인플루엔자 바이러스항체를 검출함으로써 바이러스의 감염 여부나 백신투여의 필요성 여부를 결정할 수 있다. 간접효소면역측정법에서 항원으로 사용된 재조합 HA1단백은 바큘로바이러스 발현시스템(baculovirus-expression system)을 이용하여 생산된 것이다. 총 65개의 cH3N2에 대한 양성 혹은 음성 혈청샘플을 이용하여 간접효소면역측정법으로 혈청 내의 항-cH3N2 항체를 측정한뒤 이를 HI 시험(heamagglutination inhibition test)과 비교하고 ROC(Reciever operating characteristics)방법으로 분석하였다. 모집된 혈청샘플은 cH3N2바이러스, 사람에게 감염되는 계절 인플루엔자 H3N2(A/Brisbane/10/2007), 그리고 pH1N1 인플루엔자 바이러스(A/California/04/2009)를 각각 비강으로 감염시킨 후 10일 뒤 각각의 그룹에서 혈청을 채집한 것이다. 각 혈청샘플은 교차반응의 정도를 알아보기 위한 실험도 함께 진행하였다. 흡광도 0.36을 적절한 기준 임계치로 추정하고 그 이상의 값을 각 항원에 대한 양성값으로 설정하였다. 간접효소면역측정법으로 혈청의 항원특이적 항체를 측정하여 HI시험과 비교 분석한 결과 93.5%의 민감도와 94.7%의 특이도를 보였다. 이러한 실험결과를 통해 재조합 HA1을 이용하여 cH3N2 HA1-특이적인 항체를 구하는 간접효소면역측정법이 개 혈청에서 항원특이 IgG를 측정하는데 효율적으로 사용될 수 있을 뿐만 아니라, 다량으로 실험을 수행해야 하는 상황에서는 HI시험 보다 더욱 효과적인 방법이 될 것으로 기대할 수 있을 것이다.

위 두 가지 연구결과를 종합해보면, 기도과민성반응을 일으키는 원인이 되는 2009 pH1N1 인플루엔자바이러스가 기존의 천식증상이 있는 사람에게서뿐만 아니라 천식증상이 없는 정상인에게서도 발견될 수

있으며, 마우스를 이용한 동물실험에서는 인플루엔자바이러스 감염 후 일어나는 기도과민성반응은 ILC2에 의해 유발되는 것임을 확인할 수 있다. 인플루엔자바이러스의 감염진단이나 항체의 보유유무를 진단하기 위한 방법으로 개발된 간접효소면역측정법은 HA1단백을 주축으로하여 다양한 인플루엔자바이러스 아종을 구분하고 그에 따른 항체를 측정하는 효과적인 방법임을 알려주고 있다. 이러한 방법은 백신의 효율적인 배분을 이루어 주고 그로 인해 1918 스페인 인플루엔자와 같은 대유행을 미연에 방지할 수 있는 또 하나의 방법이 될 수 있을 것으로 기대한다.

주요어: 자연면역반응, 인플루엔자A H1N1바이러스, 기도과민반응성, 선천성림프구, ILC2, type 2 사이토카인, 급성 천식, 폐 염증, 개 인플루엔자, cH3N2, 간접효소면역측정법, HA1단백

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Publication list

1. Doo Hee Shim, Young A Park, Min Jung Kim, Jung Yeon Hong, Ji Young Baek, Kyung Won Kim, Young-ho Byun, Baik-Lin Seong, Sangryeol Ryu, Manki Song, Kee-Jong Hong, Woonsung Na, Minki Hong, Daesub Song, Jeon Han Park, Myung Hyun Sohn, and Jae Myun Lee. Pandemic Influenza Virus Induces Asthmatic Symptoms via Activation of Innate Lymphoid Cell. *J Bio Chem*. Under Review
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3. Kyo Chul Koo, Doo Hee Shim, Chang Mo Yang, Saet-Byul Lee, Shi Mun Kim, Tae Young Shin, Kwang Hyun Kim, Ho Geun Yoon, Koon Ho Rha, Jae Myun Lee, Sung Joon Hong. Reduction of the $CD16^{-}CD56^{bright}$ NK Cell Subset Precedes NK Cell Dysfunction in Prostate Cancer. *Plos ONE* 2013 8(11); e78049. doi:10.1371/journal.pone.0078049
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5. Doo Hee Shim, Hyun-Jeong Ko, Gerdts Volker, Andrew A. Potter, George Mutwiri, Lorne A. Babiuk, Mi-Na Kweon. Efficacy of poly[di(sodium carboxylatophenoxy)phosphazene] (PCPP) as mucosal adjuvant to induce protective immunity against respiratory pathogens. *Vaccine* 2010 28(11) 2311-2317. doi:10.1016/j.vaccine.2009.12.069